Novel Goodpasture antigen-binding protein isoforms and protein misfolded-mediated disorders

Cross Reference

This application claims priority to U.S. Provisional Patent Application Serial Nos. 60/445,043 filed February 5, 2003; 60/445,003 filed February 5, 2003; and 60/445,004 filed February 5, 2003, which are herewith incorporated by reference in their entirety.

Background of the Invention

The conformation of the non-collagenous (NC1) domain of the α3 chain of the basement membrane collagen IV [α3(IV)NC1] depends in part on phosphorylation. Goodpasture Antigen Binding Protein (GPBP) (WO 00/50607; WO 02/061430) is a novel non-conventional protein kinase that catalyzes the conformational isomerization of the α3(IV)NC1 domain during its supramolecular assembly, resulting in the production and stabilization of multiple α3(IV)NC1 conformers in basement membranes. Elevated levels of GPBP have been associated with the production of non-tolerized α3(IV)NC1 conformers, which conduct the autoimmune response mediating Goodpasture ("GP") disease. In GP patients, autoantibodies against the non-collagenous C-terminal domain (NC1) of the type IV collagen α3 chain ("Goodpasture antigen" or "GP antigen") cause a rapidly progressive glomerulonephritis and often lung hemorrhage, the two cardinal clinical manifestations of the GP syndrome.

The identification of GPBP provided methods for identification of compounds for the treatment of autoimmune disorders, cancer, and aberrant apoptosis, and also provided potential therapeutics for these disorders. Thus, the identification of novel GPBP isoforms would be advantageous in at least these fields.

Summary of the Invention

The present invention provides novel isoforms of the Goodpasture antigen binding protein (GPBP), and related reagents, and also provides methods for isolating and detecting such novel GPBP isoforms. The invention further provides methods identifying compounds to treat

one or more of an autoimmune condition and a protein deposit-mediated disorder, as well as novel compounds and methods for treating such conditions and/or disorders.

Brief Description of the Figures

Figure 1. 91-kDa GPBP represents a non-canonical translation product of the mRNA. The cDNAs present in pcDNA3 (1), pc-n4'(2) or pc-n4'-Met_{mut} (3) were expressed in a cell-free system (in vitro) or in human 293 cells (ex vivo) and similar amounts of the corresponding mixtures or extracts were analyzed by fluorography (in vitro) or by Western blot using Mab6 antibodies (ex vivo), respectively. Unless otherwise indicated with numbers and bars we indicate in this and the following Figures the size in kDa and position of rainbow molecular weight markers from Amersham Bioscience.

Figure 2. The 91-kDa GPBP represent a non-canonical translation of the ORF existing in 5'UTR of the mRNA. In A, the ORF of the 5'-UTR of human GPBP mRNA is written in capitals and one-letter code. The 5' end and the translation direction of the indicated pcDNA3-based constructs are marked with bent arrows. The sequence of the synthetic peptide (GPBPpep2) is highlighted. In **B**, the cDNAs in the indicated constructs were expressed in a cell-free system (in vitro) or in human 293 cells (ex vivo) and analyzed as in **Fig. 1**.

Figure 3. In the cell the non-canonical 91-kDa GPBP isoform is more abundant than canonical 77-kDa. Lysates from pc-n4' (1) or from non-transfected (2,3) 293 cells were analyzed by Western blot with Mab6 (1,2) or with Mab6 and GPBPpep1 (3).

Figure 4. Cellular 91- and 120-kDa GPBP-related polypeptides are translation products of GPBP mRNA. Similar amounts (~ 50 μg) of lysates from non-transfected 293 cells (1) or from 293 cells transfected with a plasmid encoding for SiGFP (2), SiGPBP (3), SiGPBP/Δ26-1 (4) SiGPBP/Δ26-2 (5), SiGPBP/Δ26-3 (6), SiGPBP/Δ26-4 (7) were analyzed by Western blot using the indicated antibodies.

Figure 5. Localization of GPBP by subcellular fractioning of rat hepatocytes. Similar amounts (~ 50 μg) of homogenate (1), cytosol (2), microsomes (3), mitochondria (4) and lysosomes (5) isolated from rat liver were analyzed by Western blot using Mab6 antibodies. Parallel studies performed in the absence of Mab6 revealed no immunoreactive polypeptides in any of the fractions analyzed.

Figure 6. Identification of 91-kDa GPBP isoform in rat liver lysosomes and evidence for processing to 44-47-kDa isoforms. In **A**, similar amounts (~ 50 μg) of lysosomal fractions from liver of untreated (C) or leupeptin-treated (L) rats were analyzed by Western blot using Mab6 antibodies. In **B**, lysosomal fractions as in **A** were further fractioned and whole (W), soluble (S) or non-soluble (M) fractions were similarly analyzed.

Figure 7. Lysosomal proteolysis of <u>in vitro</u> expressed GPBP generates polypeptides of similar molecular mass than endogenous GPBP-related polypeptides. The cDNA in pc-n4' was expressed in a cell-free system and similar amounts of the mixtures were incubated in the absence of lysosomal extract for 20 min (1) or in the presence of lysosomal extract for 5 (2) or 20 (3) min and analyzed by SDS-PAGE and fluorography.

Figure 8. Phosphate transfer activity in isolated rat liver lysosomes. In **A**, entire (1,2,3) or broken (4,5,6) rat liver lysosomes were incubated for 0 (1,4), 10 (2,5) or 20 (3,6) min with a phosphorylation mixture containing [γ^{32}P]ATP and further analyzed by SDS-PAGE and autoradiography. In **B**, entire lysosomes from liver of untreated (Control) or leupeptin-treated (Leupeptin) rats were similarly incubated for 0 (1), 15 (2), 30 (3) or 60 (4) min and further analyzed by Western blot using Mab6 (Western) and autoradiography (^{32}P). Here and in the following Figures the autoradiographic study was performed first to avoid labeling leakage during Western blot processing. With numbers and bars we indicate the size in kDa and position of Mab6 reactive polypeptides on either study. The arrows denote the autoradiographic bands whose intensity increased during time of incubation.

Figure 9. Conformational diversification of the α3(IV)NC1 domain occurs at the endosomal-lysosomal compartment and depends on GPBP. In **A**, 293 cells expressing recombinant α3(IV)NC1 domain were treated with 20 mM NH_4Cl and/or 100 μM leupeptin. Similar amounts of serum-free media were analyzed by SDS-PAGE under reducing (R) or non-reducing (NR) conditions and Western-blot using α3(IV)NC1-specific antibodies (Mab175). In **B**, similar amounts of recombinant GPBP or α3(IV)NC1-expressing cells were incubated or cultured respectively in the absence (Con) or in the presence of the indicated GPBP modulator. Phosphorylation mixtures were analyzed as in **Fig. 8** (^{32}P) using Mab14 in the Western blot staining to determine that were not differences in the amount of recombinant protein among lanes (not shown). Culture media were analyzed as in **A** (Western).

Figure 10. GPBP interacts and phosphorylates PrP^C. In **A**, cellular extracts of cultured rat cerebellar neurons were analyzed by SDS-PAGE and Western blot using PrP (C-20) antibodies (α-PrP^C) or by far Western blot using recombinant GPBP and Mab14 (far Western). In **B**, 1 µg of bovine recombinant PrP (Prionics), human recombinant $\alpha 3$(IV)NC1 (C+) or horse heart cytochrome c from Sigma (C-) were analyzed by SDS-PAGE and Coomassie blue stained (Coomassie) or by far Western blot as in **A** (GPBP+α-GPBP). In **C**, 100 ng of human recombinant GPBP (1), same amount of GPBP with 1 µg of bovine recombinant PrP (2) or the same amount of bovine recombinant PrP (3) were separately subjected to in vitro phosphorylation and the corresponding mixtures analyzed by Western blot using PrP specific antibodies in **A** (not shown) and autoradiography (^{32}P). With an arrow we note the position of recombinant PrP.

Figure 11. PrP and GPBP interact in cells lysates. Cultured 293 cells were transfected with pc-DNA3 and pc-PrP (1), pc-DNA3 and pc-PrP^{E168R} (2), pc-Flag-n4' and pc-PrP (3) or with pc-Flag-n4' and pc-PrP^{E168R} (4), lysed and subjected to anti-FLAG immunoprecipitation. Lysates and immunoprecipitated (IP) materials were analyzed by Western blot using the indicated biotin-labeled antibodies.

Figure 12. Evidence for GPBP modulators regulating human recombinant PrP conformation in 293 cells. Human 293 cells were transfected with pc-PrP, cultured in the absence (C) or in the presence of DAB-Am-32 (D32) or DAB-Am4 (D4) and further lysed and centrifuged. The corresponding supernatants (st) and pellets were analyzed by Western blot using 3F4 anti-PrP antibodies. Similar results to those obtained with DAB-Am-32 were also observed with Q_{2D} (not shown).

Figure 13. Evidence for GPBP mRNA silencers regulating recombinant PrP conformation in 293 cells. Human 293 cells were transfected with pc-PrP and either SiGFP (C), SiGPBP/Δ26-2 (1) or SiGPBP/Δ26-4 (2) cultured for 48 h and further lysed and centrifuged. The corresponding supernatants (st) and pellets were analyzed by Western blot using 3F4 anti-PrP antibodies. Western blot analysis on the cell lysates confirmed that SiGPBP/Δ26-2 silenced endogenous GPBP more efficiently than SiGPBP/Δ26-4 (not shown).

Figure 14. Evidences for GPBP interacting with $A\beta_{1-42}$. Similar amounts (1 µg) of $A\beta_{1-42}$ (1) or GPpep1bov (2) were analyzed by far Western blot as in previous Figures. The presence of similar amounts of each of the two polypeptides in the Immobilon P membrane was

determined either with specific antibodies reacting with each polypeptide or by Ponceau S staining (not shown).

Detailed Description of the Invention

All references cited are herein incorporated by reference in their entirety.

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutshcer, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion 1998 Catalog (Ambion, Austin, TX).

As used herein, the term "GPBP" or "GPBP isoform" refers to Goodpasture antigen binding protein, and includes the various alternative GPBP isoforms disclosed herein, including GPBPΔ26 isoforms, and further includes both monomers and oligomers thereof. The various GPBP isoforms disclosed herein include 91 kDa GPBP, 77 kDa GPBP, 60 kDa GPBP, 44-47 kDa GPBP, and 32 kDa GPBP. Human, mouse, and bovine isoforms are provided herein.

As used herein, the term "GPBPΔ26" refers to Goodpasture antigen binding protein deleted for the 26 amino acid sequence shown in SEQ ID NO: 46, and the various alternative GPBP isoforms disclosed herein, and further includes both monomers and oligomers thereof. The various GPBPΔ26 isoforms disclosed herein include 91 kDa GPBPΔ26, and 77 kDa GPBPΔ26. Human, mouse, and bovine isoforms are provided herein.

As used herein, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise. For example, reference to a "GPBP isoform" means one or more GPBP isoforms.

As used herein the term "non-canonical" means that the GPBP being referred to is not expressed from the methionine initiation codon that yields 77 kDa GPBP or 77 kDa GPBPΔ26.

For the sake of simplicity, recitations of "non-canonical GPBP" include both non-canonical GPBP isoforms and non-canonical GPBPΔ26 isoforms.

As used herein a "protein deposit-mediated disorder" means a disease mediated by abnormal deposition of a specific protein, including but not limited to Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, prion diseases, type II diabetes, and autoimmune disorders. The protein deposit may be amyloid matter or para-amyloid matter.

As used herein an "autoimmune condition" is selected from the group consisting of Goodpasture Syndrome, multiple sclerosis, systemic lupus erythematosus, cutaneous lupus erythematosus, pemphigus, pemphigoid and lichen planus.

The amino acid sequence of 77 kDa GPBP and GPBPΔ26 was disclosed in US Patent No. 6579969 Issued June 17, 2003 and corresponding PCT publication WO 00/50607, published August 31, 2000. GPBP was identified therein as a 71 kDa protein that underwent post-translational modification to result in higher molecular weight polypeptides. It was also disclosed that the 71 kDa protein began at a methionine residue, but that in the 5' untranslated region upstream of the coding region encoding the amino-terminal methionine of the 71 kDa protein, the cDNA clone encoding 71 kDa GPBP contained an open reading frame without an initiation codon for translation. It was speculated that an mRNA editing process inserting a single base pair (U) might generate an operative in-frame start site and an ORF of 754-residues containing an export signal immediately downstream of the edited Met.

The present invention demonstrates that, rather than the mRNA editing process speculated on in WO 00/50607, the human GPBP mRNA undergoes non-canonical translation initiation to produce a 91-kDa isoform of GPBP (91 kDa GPBP). The resulting protein product is not the 753 amino acid residue protein speculated upon in WO 00/50607, but is believed to be a protein of approximately 727 amino acid residues comprising the amino acid sequence of SEQ ID NO:6. The corresponding predicted 91 kDa GPBPΔ26 amino acid sequence comprises the amino acid sequence of SEQ ID NO:8. The present invention also provides mouse and bovine homologs of the human 91 kDa polypeptide: mouse 91 kDa GPBP (SEQ ID NO:94), mouse 91 kD GPBPΔ26 (SEQ ID NO:96), bovine 91 kDa GPBP (SEQ ID NO:98), and bovine 91 kDa GPBPΔ26 (SEQ ID NO:100).

For the sake of simplicity, the different isoforms are referred to as being the same molecular weight, whether a GPBP isoform or a GPBPΔ26 isoform. It will be apparent to one of

skill in the art that the GPBPΔ26 isoform will contain 26 fewer amino acid residues than the corresponding GPBP isoform, and thus will have a molecular weight approximately 2.6 kDa less than the corresponding GPBP isoform.

The present invention further demonstrates that various processed forms of these GPBP isoforms exist, and provides evidence for the dependency of their subcellular localization on the particular processing event that occurs. The invention further provides a series of truncation mutants of the GPBP cDNA that are predicted to encode the primary sequence signals to direct their differential subcellular localization patterns. The expression products of these truncation mutants are as follows (also, see Figure 2):

Δ102 GPBP	SEQ ID NO:26
Δ102 GPBPΔ26	SEQ ID NO:28
Δ174 GPBP	SEQ ID NO:22
Δ174 GPBPΔ26	SEQ ID NO:24
Δ246 GPBP	SEQ ID NO:18
Δ246 GPBPΔ26	SEQ ID NO:20
Δ315 GPBP	SEQ ID NO:14
Δ315 GPBPΔ26	SEQ ID NO:16
Δ369 GPBP	SEQ ID NO:10
Δ369 GPBPΔ26	SEQ ID NO:12

Thus, in one aspect, the present invention provides substantially purified polypeptide comprising or consisting of an amino acid sequence according to SEQ ID NO:29, which is the amino acid sequence present in Δ369 GPBP (or Δ369 GPBPΔ26) that is not present in GPBP (or GPBPΔ26). The amino acid sequence of SEQ ID NO:29 is GAGAGLLLGCRAS. In one embodiment of this aspect, the substantially purified polypeptides comprise or consist of the amino acid sequence of SEQ ID NO:30, which is the amino acid sequence present in Δ315 GPBP (or Δ315 GPBPΔ26) that is not present in GPBP (or GPBPΔ26).

In a further embodiment of this aspect, the substantially purified polypeptides comprise or consist of the amino acid sequence of SEQ ID NO:31, which is the amino acid sequence present in Δ246 GPBP (or Δ246 GPBPΔ26) that is not present in GPBP (or GPBPΔ26). In a further

embodiment of this aspect, the substantially purified polypeptides comprise or consist of the amino acid sequence of SEQ ID NO:32, which is the amino acid sequence present in Δ174 GPBP (or Δ174 GPBPΔ26) that is not present in GPBP (or GPBPΔ26).

In a further embodiment of this aspect, the substantially purified polypeptides comprise or consist of the amino acid sequence of SEQ ID NO:33, which is the amino acid sequence present in Δ102 GPBP (or Δ102 GPBPΔ26) that is not present in GPBP (or GPBPΔ26). In a further embodiment of this aspect, the substantially purified polypeptides comprise or consist of the amino acid sequence of SEQ ID NO:34, which is the predicted amino acid sequence present in 91 kDa GPBP (or 91 kDa GPBPΔ26) that is not present in GPBP (or GPBPΔ26).

In various further embodiments of this aspect of the invention, the substantially purified polypeptides comprise or consist of an amino acid sequence selected from the group consisting of SEQ ID NO:6 (predicted 91 kDa GPBP), SEQ ID NO:8 (predicted 91 kDa GPBPΔ26), SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:98 (bovine 91 kD GPBP homolog), and SEQ ID NO:100 (bovine 91 kD GPBPΔ26 homolog).

In a further aspect, the present invention provides substantially purified polypeptides comprising or consisting of an amino acid sequence according to SEQ ID NO:101 (GAGAGLLLGCR<u>V</u>S), which is present in mouse and rat GPBP isoforms, and which corrsponds to SEQ ID NO:29 from the human sequence but differs in a single amino acid residue (underlined). Sequence comparison of potential open reading frames in the mouse, bovine, and rat GPBP mRNA indicates that they encode sequences that are of great similarity to the human GPBP isoforms disclosed herein: at least 94% identity for the 91 kDa GPBP between human, mouse, and bovine homolgs and at least 81% identity between human, mouse, rat and bovine homolgs for the predicted amino acid sequences upstratem of canonical GPBP. Thus, in another embodiment of this aspect, the present invention provides substantially purified polypeptides comprising an amino acid sequence that are at least 80% identical to SEQ ID NO:34. Such sequence identity is as determined using the BLAST engine for local alignment. The stand-alone executable for blasting two sequences (bl2seq) can be retrieved from the NCBI internet site, and is also disclosed in FEMS Microbiol Lett. 174:247-250 (1999).

In another embodiment of this aspect, the present invention provides substantially purified polypeptides comprising or consisting of an amino acid sequence selected from the group consisting

of SEQ ID NO:94 and SEQ ID NO:96. These polypeptides represent mouse homologs of human 91 kDa GPBP and 91 kDa GPBPΔ26, respectively.

In these various aspects and embodiments, the present invention provides novel polypeptides that can be used to generate antibodies to distinguish between different GPBP isoforms, and which can also be used, for example, as tools to identify candidate compounds for inhibiting various specific types of GPBP isoforms and also to identify candidate compounds for treating autoimmunity and amyloidosis disorders, as discussed in more detail below.

As used herein, the term "substantially purified" means that the protein has been separated from its in vivo cellular environments. Thus, the protein can either be purified from natural sources, or recombinant protein can be purified from the transfected host cells disclosed above. In a preferred embodiment, the proteins are produced by the transfected cells disclosed above, and purified using standard techniques. (See for example, *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press.)) The protein can thus be purified from prokaryotic or eukaryotic sources. In various further preferred embodiments, the protein is purified from bacterial, yeast, or mammalian cells. In a preferred embodiment, substantially purified means that the polypeptide is substantially free of gel agents, such as polyacrylamide and agarose. In a further preferred embodiment, "substantially purified" means that they are free of other GPBP isoforms. In a further preferred embodiment, the substantially purified proteins are present in solution. As used herein, the term "substantially free of other proteins" means that contaminating proteins make up no more than about 5% of the substantially purified sample, preferably no more than about 3%.

In another embodiment of this aspect of the invention, the substantially purified polypeptide comprises or consists of an amino acid sequence according to the genus R1-R2-R3, wherein

R1 is 0-90 amino acids of SEQ ID NO:35;

R2 is the amino acid sequence according to SEQ ID NO:29; and

R3 is an amino acid sequence selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO:4.

In this embodiment, the R1 position is variable, and can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38. 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, or 90

amino acids of SEQ ID NO:35. If R1 is 90 amino acid residues of SEQ ID NO:35 the resulting polypeptide comprises the polypeptide of SEQ ID NO:6 or SEQ ID NO:8, depending on the identity of the R3 group. Based on the above teachings, the various polypeptides encompassed by this R1 embodiment will be apparent to one of skill in the art.

In another embodiment, the substantially purified polypeptide comprises or consists of a polypeptide of the genus X1-X2, wherein:

X1 is 0-90 amino acids of SEQ ID NO:35;

X2 is the amino acid sequence according to SEQ ID NO:29

wherein the polypeptide does not include the sequence of SEQ ID NO:2 or SEQ ID NO:4.

In this embodiment, the R1 position is variable, and can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38. 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, or 90 amino acids of SEQ ID NO:35. If R1 is 90 amino acid residues of SEQ ID NO:35, the resulting polypeptide comprises the polypeptide of SEQ ID NO:34. Based on the above teachings, the various polypeptides encompassed by this R1 embodiment will be apparent to one of skill in the art.

In this embodiment, the substantially purified polypeptides provide tools to distinguish between the different isoforms of GPBP identified herein. For example, the substantially purified polypeptides according to this embodiment can be used to generate antibodies that selectively bind to the 91 kDa GPBP and that do not bind to the 77 kDa GPBP. Such antibodies will be of utility, for example, in immunodetection assays as described below.

The substantially purified polypeptides of the invention can be made by any method known to those of skill in the art, but are preferably made by recombinant means based on the teachings provided herein. For example, a coding region of interest as disclosed herein can be cloned into a recombinant expression vector, which can then be used to transfect a host cell for recombinant protein production by the host cells.

"Recombinant expression vector" includes vectors that operatively link a nucleic acid coding region or gene to any promoter capable of effecting expression of the gene product. The promoter sequence used to drive expression of the disclosed nucleic acid sequences in a mammalian system may be constitutive (driven by any of a variety of promoters, including but not limited to, CMV, SV40, RSV, actin, EF) or inducible (driven by any of a number of

inducible promoters including, but not limited to, tetracycline, ecdysone, steroid-responsive). The construction of expression vectors for use in transfecting prokaryotic cells is also well known in the art, and thus can be accomplished via standard techniques. (See, for example, Sambrook, Fritsch, and Maniatis, in: Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989; *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion 1998 Catalog (Ambion, Austin, TX)

The expression vector must be replicable in the host organisms either as an episome or by integration into host chromosomal DNA. In a preferred embodiment, the expression vector comprises a plasmid. However, the invention is intended to include other expression vectors that serve equivalent functions, such as viral vectors.

The protein may comprise additional sequences useful for promoting purification of the protein, such as epitope tags and transport signals. Examples of such epitope tags include, but are not limited to FLAG (Sigma Chemical, St. Louis, MO), myc (9E10) (Invitrogen, Carlsbad, CA), 6-His (Invitrogen; Novagen, Madison, WI), and HA (Boehringer Manheim Biochemicals). Examples of such transport signals include, but are not limited to, export signals, secretory signals, nuclear localization signals, and plasma membrane localization signals.

As disclosed below, the inventors have further discovered that at least the 91-kDa GPBP enters into the cell secretory pathway, reaches the endosomal/lysosomal compartment and undergoes proteolysis to yield products of lower molecular mass. Thus, in another embodiment, the polypeptides of the present invention are substantially purified processed GPBP polypeptides derived from a precursor polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and/or SEQ ID NO:8 wherein the substantially purified polypeptide is reactive with an antibody selective for one or more epitopes within one or more of the GPBP isoforms disclosed herein, wherein the substantially purified processed GPBP polypeptide is selected from the group consisting of:

(a) a 60 kDa GPBP with a molecular weight of approximately 60 kDa in denaturing gel electrophoresis, wherein the 60 kDa GPBP is present in lysosomes, cytoplasm, microsomes, and mitochondria in liver tissue, wherein the 60 kDa GPBP is membrane-associated or soluble in the lysosomes in liver tissue;

(b) a 44-47 kDa GPBP with a molecular weight of approximately 44-47 kDa in denaturing gel electrophoresis, wherein the 44-47 kDa GPBP is present in lysosomes in liver tissue, wherein the 44-47 kDa GPBP is predominately formed through a leupeptin-sensitive proteolysis in liver tissue;

(c) a 32 kDa GPBP with a molecular weight of approximately 32 kDa in denaturing gel electrophoresis, wherein the 32 kDa GPBP is present in cytoplasm, mitochondria, microsomes, and lysosomes in liver tissue, and wherein the 32 kDa GPBP is formed through a leupeptin-insensitive proteolysis in liver lysosomes.

As used herein, being of an approximate molecular weight as determined by denaturing gel electrophoresis means that the polypeptide is within 0-10% of the recited molecular weight, more preferably within 0-5%, and even more preferably within 0-3% under the following gel conditions

As used herein, determination of molecular weights is as would be determined under the following conditions: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) performed on MiniProtean III (Bio-Rad) using polyacrylamide gels (29.2:0.8 acrylamide:bisacrylamide) at room temperature and constant voltage (200 volts); running buffer of 192 mM glycine, 24.7 mM Tris and 1% SDS; stacking gel of 3.65% acrylamide/bisacrylamide 122 mM HCl-Tris pH 6.8 , 0.1% SDS, 0.146% ammonium persulfate, 0.146% Temed; running gel of 10% acrylamide/bisacrylamide, 373 mM HCl-Tris pH 8.8;0.1% SDS, 0.1 % ammonium persulfate, 0.1 % Temed; and samples were 31.25 mM HCl-Tris pH 6.8, 5.16% glycerol, 1% SDS and 2.5 % β-mercaptoethanol.

This range represents a standard fluctuation for such molecular weight determinations based on differences in gel reagents, running time, temperature, and voltage, and other variables as would be recognized by those of skill in the art.

As used herein, the recitation of a processed GPBP being in a specific subcellular compartment in liver tissue means that the protein is present in detectable levels in the recited cellular compartment, and does not mean that it is not present in detectable levels in other cellular compartments.

As used herein, being "membrane-associated" means that, in extracts of the subcellular extract being analyzed, detectable levels of the polypeptide of interest are found in the membrane fraction in subcellular fractions isolated according to the methods disclosed below.

As used herein, "leupeptin-sensitive" means that, in the presence of sufficient quantities of leupeptin, production of the recited proteolytic product is reduced. As used herein, "leupeptin-insensitive" means that, in the presence of similar quantities of leupeptin as above, production of the recited proteolytic product is not reduced. Preferred embodiments for determining leupeptin-sensitivity are as described below in the experimental section.

The substantially purified processed GPBP polypeptides of this embodiment can be produced, for example, by a method comprising (a) providing cells that express one or more polypeptide comprising or consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8; (b) lysing the cells and isolating one or more fractions of the cells comprising fractions selected from the group consisting of cytoplasmic-containing fractions, mitochondrial-containing fractions, microsomal-containing fractions, and lysosomal-containing fractions; (c) contacting the isolated fractions with an immunoaffinity column comprising an antibody that selectively binds to a polypeptide comprising or consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35 under conditions that result in binding of one or more of the 60 kDa GPBP, the 44-47 kDa GPBP, and the 32 kDa GPBP to the immunoaffinity column; (d) washing the column under conditions that remove cellular contents that do not selectively bind to the immunoafinity column; (e) eluting the bound material from the immunoaffinity column to provide an eluate; and (f) size fractionating the eluate and isolating one or more of the fractions consisting of the approximately 60 kDa fraction, the approximately 44-47 kDa fraction, and the approximately 32 kDa fraction, wherein the approximately 60 kDa fraction contains the substantially purified 60 kDa GPBP; the approximately 44-47 kDa fraction contains the substantially purified 44-47 kDa GPBP, and the approximately 32 kDa fraction contains the substantially purified 32 kDa GPBP.

In a preferred embodiment of this method, the cells express at least one polypeptide comprising or consisting of an amino acid sequence selected from the group consisting of SEQ ID NO: 6 and SEQ ID NO:8, more preferably SEQ ID NO:6.

Antibodies for use in these methods include those described herein as well as in WO 00/50607 and WO 02/061430. Cell fractionation, immunoaffinity column chromatography, size fractionation, and suitable wash and elution conditions are known to those of skill in the art.

In another embodiment, the substantially purified processed GPBP polypeptides of this embodiment can be produced by a method comprising (a) providing cells that express one or more recombinant polypeptides comprising or consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8; (b) lysing the cells and obtaining a partially purified cell extract containing the recombinant polypeptides; (c) contacting the partially purified cell extract with liver lysosomal extracts under conditions that promote processing of the recombinant polypeptides to produce a processed extract; (d) contacting the processed extract with an immunoaffinity column comprising an antibody that selectively binds to an epitope within the recombinant polypeptides and/or their processed forms comprising or consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35 under conditions that result in binding of recombinant polypeptides and their processed forms to the immunoaffinity column; (e) washing the column under conditions that remove cellular contents that do not selectively bind to the immunoafinity column; (f) eluting the bound material from the immunoaffinity column to provide an eluate; and (g) size fractionating the eluate and isolating one or more of the fractions consisting of the approximately 60 kDa fraction, the approximately 44-47 kDa fraction, and the approximately 32 kDa fraction, wherein the approximately 60 kDa fraction contains the substantially purified 60 kDa GPBP; the approximately 44-47 kDa fraction contains the substantially purified 44-47 kDa GPBP, and the approximately 32 kDa fraction contains the substantially purified 32 kDa GPBP.

In a preferred embodiment of this method, the cells express at least one polypeptide comprising or consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:6 and SEQ ID NO:8, more preferably SEQ ID NO:6.

For this embodiment, one of skill in the art can use the teachings of the invention to prepare recombinant expression vectors expressing the recited polypeptides. Preparing cell extracts and liver lysosomal extracts are known to those in the art, and are further described below.

In a further embodiment, the polypeptides of the present invention include an isolated polypeptide consisting of the amino acid sequence of SEQ ID NO:38 (AA 1-299 of SEQ ID NO:2). This polypeptide is a truncated version of the 77 kDa GPBP. As described below, this polypeptide is demonstrated to have a greater kinase activity under acidic conditions than GPBP, and thus may be functionally similar to the GPBP forms present in the lysosome.

In a further aspect, the present invention provides pharmaceutical compositions comprising one or more substantially purified polypeptide as described above and a pharmaceutically acceptable carrier. In a non-limiting example, the pharmaceutical compositions of this aspect of the invention can be used for immunization to prepare antibodies specific for non-canonical GPBP isoforms, which themselves can be used as therapeutics to modulate GPBP activity. Alternatively, the pharmaceutical compositions according to this aspect of the invention can themselves be used as therapeutics to inhibit GPBP activity in a subject in need thereof.

In another aspect, the present invention provides antibodies that selectively bind to the substantially purified polypeptides disclosed herein, but which do not selectively bind to the peptide sequence PRSARCQARRRRGGRTSS (SEQ ID NO:103).

In a preferred embodiment, the antibodies of the invention selectively bind to an epitope present within the GPBP isoforms disclosed herein and do not selectively bind to a polypeptide consisting of the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4. In this embodiment, it is further preferred that the antibodies selectively bind to one or more proteins comprising or consisting of a sequence selected from the group consisting of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, and/or to an epitope within one or more polypeptides selected from the group consisting of the 60 kDa GPBP, the 44-47 kDa GPBP, and the 32 kDa GPBP. Such antibodies can be produced by immunization of a host animal with either the complete GPBP isoforms disclosed herein or with antigenic peptides thereof, while selecting against those that selectively bind to SEQ ID NO:103, and/or to SEQ ID NO:2 and/or SEQ ID NO:4 (via, for example, adsorption of such antibodies on an affinity column comprising the polypeptide of SEQ ID NO:103, SEQ ID NO:2 and/or SEQ ID NO:4). In a preferred embodiment, the antibodies selectively bind to an epitope within an amino acid sequence selected from the group consisting of SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID

NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35. These sequences are not included in the sequence of SEQ ID NO:2 or SEQ ID NO:4, and thus antibodies directed against epitopes within these sequences do not selectively bind to SEQ ID NO:2 or SEQ ID NO:4. Suitable antibodies include polyclonal, monoclonal, and humanized monoclonal antibodies.

In a further embodiment, the antibodies selectively bind to an isolated polypeptide consisting of the amino acid sequence of SEQ ID NO:38 (AA 1-299 of SEQ ID NO:2 or SEQ ID NO:4). This polypeptide is a truncated version of the 77 kDa GPBP or GPBPΔ26. As described below, this polypeptide is demonstrated to have a greater kinase activity under acidic conditions than GPBP, and thus may be functionally similar to the GPBP forms present in the lysosome.

As used herein, the term "selectively bind(s)" means that the antibodies preferentially bind to the polypeptide in question in a mixture of polypeptides.

In a further aspect, the present invention provides methods for making antibodies selective for one or more GPBP isoforms, comprising immunizing a host animal with an antigenic epitope derived from a polypeptide consisting of an amino sequence selected from the group consisting of SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, and SEQ ID NO:101 and isolating antibodies from the host animal that selectively bind to the polypeptide, wherein the isolated antibodies are selective for one or more Goodpasture antigen binding protein isoforms.

Antibodies can be made by well-known methods, such as described in Harlow and Lane, Antibodies; A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1988). In one example, preimmune serum is collected prior to the first immunization. A substantially purified polypeptide of the invention, or antigenic fragments thereof, together with an appropriate adjuvant, are injected into an animal in an amount and at intervals sufficient to elicit an immune response. Animals are bled at regular intervals, preferably weekly, to determine antibody titer. The animals may or may not receive booster injections following the initial immunization. At about 7 days after each booster immunization, or about weekly after a single immunization, the animals are bled, the serum collected, and aliquots are stored at about -20° C. Polyclonal antibodies against the proteins and peptides of the invention can then be purified directly by passing serum collected from the animal through a column to which non-antigen-related proteins prepared from the same expression system without GPBP-related proteins bound.

Monoclonal antibodies can be produced by obtaining spleen cells from the animal. (See Kohler and Milstein, Nature 256, 495-497 (1975)). In one example, monoclonal antibodies (mAb) of interest are prepared by immunizing inbred mice with the proteins or peptides of the invention, or an antigenic fragment thereof. The mice are immunized by the IP or SC route in an amount and at intervals sufficient to elicit an immune response. The mice receive an initial immunization on day 0 and are rested for about 3 to about 30 weeks. Immunized mice are given one or more booster immunizations of by the intravenous (IV) route. Lymphocytes, from antibody positive mice are obtained by removing spleens from immunized mice by standard procedures known in the art. Hybridoma cells are produced by mixing the splenic lymphocytes with an appropriate fusion partner under conditions which will allow the formation of stable hybridomas. The antibody producing cells and fusion partner cells are fused in polyethylene glycol at concentrations from about 30% to about 50%. Fused hybridoma cells are selected by growth in hypoxanthine, thymidine and aminopterin supplemented Dulbecco's Modified Eagles Medium (DMEM) by procedures known in the art. Supernatant fluids are collected from growth positive wells and are screened for antibody production by an immunoassay such as solid phase immunoradioassay. Hybridoma cells from antibody positive wells are cloned by a technique such as the soft agar technique of MacPherson, Soft Agar Techniques, in Tissue Culture Methods and Applications, Kruse and Paterson, Eds., Academic Press, 1973.

"Humanized antibody" refers to antibodies derived from a non-human antibody, such as a mouse monoclonal antibody. Alternatively, humanized antibodies can be derived from chimeric antibodies that retains or substantially retains the antigen-binding properties of the parental, non-human, antibody but which exhibits diminished immunogenicity as compared to the parental antibody when administered to humans. For example, chimeric antibodies can comprise human and murine antibody fragments, generally human constant and mouse variable regions. Since humanized antibodies are far less immunogenic in humans than the non-human monoclonal antibodies, they are preferred for therapeutic antibody use.

Humanized antibodies can be prepared using a variety of methods known in the art, including but not limited to (1) grafting complementarity determining regions from a non-human monoclonal antibody onto a human framework and constant region ("humanizing"), and (2) transplanting the non-human monoclonal antibody variable domains, but "cloaking" them with a human-like surface by replacement of surface residues ("veneering"). These methods are

disclosed, for example, in, e.g., Jones et al., Nature 321:522-525 (1986); Morrison et al., Proc. Natl. Acad. Sci., U.S.A., 81:6851-6855 (1984); Morrison and Oi, Adv. Immunol., 44:65-92 (1988); Verhoeyer et al., Science 239:1534-1536 (1988); Padlan, Molec. Immun. 28:489-498 (1991); Padlan, Molec. Immunol. 31(3):169-217 (1994); and Kettleborough, C. A. et al., Protein Eng. 4(7):773-83 (1991).

To generate an antibody response, the polypeptides of the present invention are typically formulated with a pharmaceutically acceptable carrier for parenteral administration. Such acceptable adjuvants include, but are not limited to, Freund's complete, Freund's incomplete, alum-precipitate, water in oil emulsion containing Corynebacterium parvum and tRNA. The formulation of such compositions, including the concentration of the polypeptide and the selection of the vehicle and other components, is within the skill of the art.

The term antibody as used herein is intended to include antibody fragments thereof which are selectively reactive with the polypeptides of the invention, or fragments thereof. Antibodies can be fragmented using conventional techniques, and the fragments screened for utility in the same manner as described above for whole antibodies. For example, $F(ab')_2$ fragments can be generated by treating antibody with pepsin. The resulting $F(ab')_2$ fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

In a further aspect, the invention provides methods for detecting the presence of one or more of the polypeptides of the invention in a protein sample, comprising providing a protein sample to be screened, contacting the protein sample to be screened with an antibody against one or more of the polypeptides of the invention, and detecting the formation of antibody-antigen complexes. In a preferred embodiment, methods for detecting the presence of a protein that is substantially similar to one or more polypeptides comprising or consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, and/or a protein that is substantially similar to one or more polypeptides selected from the group consisting of 60 kDa GPBP, 44-47 kDa GPBP, and 32 kDa GPBP comprise

a) providing a protein sample to be screened;

b) contacting the protein sample to be screened with an antibody selective for one or more of the GPBP isoforms disclosed herein under conditions that promote antibody-antigen complex formation; and

c) detecting the formation of antibody-antigen complexes, wherein the presence of the antibody-antigen complex indicates the presence of a protein comprising or consisting of a sequence that is substantially similar to a sequence selected from the group consisting of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, and/or a protein that is substantially similar to one or more polypeptides selected from the group consisting of 60 kDa GPBP, 44-47 kDa GPBP, and 32 kDa GPBP.

As used herein, the term "substantially similar" means that the polypeptides share at least 70% amino acid identity along their co-linear portions, and more preferably 75%, 80%, 85%, 90%, or 95% identity.

The antibody can be polyclonal, monoclonal, or humanized monoclonal as described above, although monoclonal antibodies are preferred. As used herein, the term "protein sample" refers to any sample that may contain the polypeptides of the invention, and fragments thereof, including but not limited to tissues and portions thereof, tissue sections, intact cells, cell extracts, purified or partially purified protein samples, bodily fluids, and nucleic acid expression libraries. Accordingly, this aspect of the present invention may be used to test for the presence of the non-canonical GPBP isoforms disclosed herein in these various protein samples by standard techniques including, but not limited to, immunolocalization, immunofluorescence analysis, Western blot analysis, ELISAs, and nucleic acid expression library screening, (See for example, Sambrook et al, 1989.) In one embodiment, the techniques may determine only the presence or absence of the protein or peptide of interest. Alternatively, the techniques may be quantitative, and provide information about the relative amount of the protein or peptide of interest in the sample. For quantitative purposes, ELISAs are preferred.

Detection of immunocomplex formation between the polypeptides of the invention, and their antibodies or fragments thereof, can be accomplished by standard detection techniques. For example, detection of immunocomplexes can be accomplished by using labeled antibodies or secondary antibodies. Such methods, including the choice of label are known to those ordinarily

skilled in the art. (Harlow and Lane, Supra). Alternatively, the antibodies can be coupled to a detectable substance. The term "coupled" is used to mean that the detectable substance is physically linked to the antibody. Suitable detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase. Examples of suitable prosthetic-group complexes include streptavidin/biotin and avidin/biotin. Examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin. An example of a luminescent material includes luminol. Examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S or ^{3}H.

Such methods of detection are useful for a variety of purposes, including but not limited to detecting an autoimmune condition, identifying cells targeted for or undergoing apoptosis, immunolocalization of the proteins of interest in a tissue sample, Western blot analysis, and screening of expression libraries to find related proteins.

In another aspect, the present invention provides isolated nucleic acids that encode the truncated GPBP polypeptides of the invention. In one embodiment, the isolated nucleic acids consist of sequences selected from the group consisting of SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, and SEQ ID NO:27.

The isolated nucleic acid sequence may comprise RNA or DNA. As used herein, "isolated nucleic acids" are those that have been removed from their normal surrounding nucleic acid sequences in the genome or in cDNA sequences. Such isolated nucleic acid sequences may comprise additional sequences useful for promoting expression and/or purification of the encoded protein, including but not limited to polyA sequences, modified Kozak sequences, and sequences encoding epitope tags, export signals, and secretory signals, nuclear localization signals, and plasma membrane localization signals.

In another aspect, the present invention provides recombinant expression vectors comprising isolated nucleic acids consisting of a sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, and SEQ ID NO:27. Recombinant expression vectors are vectors that operatively link a nucleic acid coding region or gene to any promoter capable of effecting

expression of the gene product that are operably linked to a promoter, and are discussed in more detail above.

In a further aspect, the present invention provides host cells that have been transfected with the recombinant expression vectors disclosed herein, wherein the host cells can be either prokaryotic or eukaryotic. The cells can be transiently or stably transfected. Such transfection of expression vectors into prokaryotic and eukaryotic cells can be accomplished via any technique known in the art, including but not limited to standard bacterial transformations, calcium phosphate co-precipitation, electroporation, or liposome mediated-, DEAE dextran mediated-, polycationic mediated-, or viral mediated transfection. (See, for example, Molecular Cloning: A Laboratory Manual (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press; Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed. (R.I. Freshney. 1987. Liss, Inc. New York, NY).

Methods for identifying and making candidate compounds to treat autoimmune conditions and/or protein deposit-mediated disorders

GPBP displays a number of biological features to be considered a good candidate as a pivotal component of the cellular machinery catalyzing conformational isomerization and supramolecular assembly of autoantigens and inducing immune response during autoimmune pathogenesis (See below, as well as WO 00/50607; WO 02/061430). The results disclosed herein suggest that GPBP is an integral component of the endosomal-lysosomal pathway which activity is regulated in part by a catepsin-dependent processing, a biological strategy described for other enzymes (Pham, C. T., & T. J. Ley, (1999). Proc Natl Acad Sci U S A 96(15): 8627-8632). These proteases are critical in processing proteins entering endosomal pathway and producing peptides that are presented through MHC class II (Chapman, H. A., (1998) Curr Opin Immunol 10(1): 93-102). Disturbance of lysosomal environment in a more general manner such as modifying the pH using compounds as chloroquine or in a more specific manner using catepsin inhibitors such as leupeptin has been shown to alter peptide presentation by MHC class II (Demotz, S., P. M. Matricardi, C. Irle, P. Panina, A. Lanzavecchia, & G. Corradin, (1989) J Immunol 143(12): 3881-3886; Turk, V., B. Turk, & D. Turk, (2001) EMBO J 20(17): 4629-4633). We have shown herein that leupeptin treatment substantially alters lysosomal processing of GPBP and therefore also likely induces an alteration in GPBP activity, which in turn suggests

that altered peptide presentation and altered GPBP activity may be related and perhaps critical in autoimmune pathogenesis, which necessarily requires aberrant peptide presentation to be effective.

A feature common to many degenerative diseases is the formation of deposits of specific polypeptides. Where and how these deposits appear is highly specific and tightly related with pathogenesis. The deposits can be nuclear inclusion bodies, as in cerebellar ataxia, or be at the ER lumen, such as in some degenerative disease affecting liver and neurons, or be cytoplasmic inclusion bodies, as in Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis; or endosomal-lysosomal, as in Alzheimer's disease, prion diseases, and type II diabetes. GPBP is an ubiquitous protein that has been independently related to conformational catalysis of substrate proteins (WO 00/50607; WO 02/061430) and in the formation of protein deposits in animal models that develop a degenerative nephropaty associated to an autoimmune response. Consequently the finding disclosed herein that GPBP interacts with PrP and $A\beta_{1-42}$ two polypeptides that undergo conformational alteration and form amyloid deposits in prion diseases and Alzheimer's disease, respectively, represents strong evidence for GPBP being involved in the pathogenesis of these degenerative diseases. More specifically, a protein resident in the endosomal-lysosomal pathway named Protein X has been proposed to bind to PrP and catalyze the conformational transition from PrP^{C} to Prp^{Sc} (Prusiner, S. B., (1998). "Prions." Proc Natl Acad Sci U S A 95(23): 13363-13383.). Herein we present evidence demonstrating that GPBP binds to PrP in a Protein X fashion, phosphorylates PrP, forms aggregates with it and, as a consequence of this interaction, PrP undergoes conformational changes that renders PrP highly insoluble and precipitable. To our knowledge, GPBP represents the best molecular candidate to be Protein X in prion diseases as well as to perform a similar role in other protein deposit-mediated human disease.

Thus, in another aspect, the present invention provides methods for identifying compounds to treat an autoimmune disorder, wherein the method comprises identifying compounds that inhibit activity of one or more GPBP isoforms of the present invention. In a preferred embodiment, the one or more GPBP isoform comprises or consists of a sequence selected from the group consisting of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, or one or more GPBP isoforms selected from the group

consisting of 60 kDa GPBP, 44-47 kDa GPBP, and 32 kDa GPBP, wherein such compounds are candidate compounds for treating an autoimmune condition and/or protein deposit-mediated disorders.

In another aspect, the present invention provides methods for identifying compounds to treat a protein deposit-mediated condition, wherein the method comprises identifying compounds that inhibit activity of one or more GPBP isoforms of the present invention. In a preferred embodiment, the one or more GPBP isoforms comprises or consists of a sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, or are selected from the group consisting of 60 kDa GPBP, 44-47 kDa GPBP, and 32 kDa GPBP, wherein such compounds are candidate compounds for treating an autoimmune condition and/or protein deposit-mediated disorders.

In a further embodiment of these methods, the method further comprises making the compounds identifed as candidate compounds for treating an autoimmune condition and/or protein deposit-mediated disorders. In one example, such compounds are organic molecules that are made using standard chemical methods. In another example, such compounds are polypeptides, which are made by methods disclosed herein for making polypeptides.

In one embodiment of these aspects, the method comprises identifying compounds that inhibit GPBP kinase activity. Such inhibition can be inhibition of GPBP autophosphorylation and/or inhibition of GPBP phosphorylation of target polypeptide, such as α3(IV) NC1 domain, myelin basic protein, and prion protein. Examples of such target polypeptides comprise those provided as SEQ ID NO:52 (α3(IV)NC1); SEQ ID NO:53 (MBP); and SEQ ID NO:54 (PrP), or functional equivalents. In a further embodiment, the method comprises identifying compounds that inhibit GPBP catalysis of conformational isomerization of a target polypeptide, such as α3(IV) NC1 domain, myelin basic protein, prion protein, and $A\beta_{1-42}$. Examples of such polypeptides are as described above, also polypeptides comprising SEQ ID NO:55 ($A\beta_{1-42}$), and functional equivalents thereof. Those of skill in the art will be able to identify other target polypeptides that can be used in the methods of the invention, such as SEQ ID NO:102, a functional equivalent for MBP.

In a further embodiment of these aspects, the method comprises identifying compounds that inhibit both GPBP kinase activity and GPBP catalysis of conformational isomerization of a target polypeptide.

The phosphorylation assays can be conducted in vitro on isolated targets, or can comprise analyzing the effects of the one or more test compounds on phosphorylation in cultured cells, although in vitro assays are preferred. A preferred method for identifying compounds that reduce in vitro phosphorylation of the target polypeptide comprises incubating a target polypeptide and ATP in vitro in the presence or absence of one or more test compounds under conditions that promote phosphorylation of the target polypeptide in the absence of the one or more test compounds; detecting phosphorylation of the target polypeptide; and identifying test compounds that reduce phosphorylation of the target polypeptide relative to phosphorylation of the target polypeptide in the absence of the one or more test compounds.

One of skill in the art is capable of determining suitable phosphorylation conditions for conducting the phosphorylation assay, and thus the present method is not limited by the details of the particular phosphorylation conditions employed. A non-limiting example of such suitable conditions for assaying phosphorylation of the first target comprises the use of 25 mM β-glycerol phosphate pH 7, 0.5 mM EGTA, 8 mM Mg Cl_2, 5 mM $MnCl_2$, 1 mM DTT, y 0.132 μM [$\gamma^{32}P$]-ATP using 100-200 ng of enzyme and 1 μg of substrate at variable time at 30° C.

In one embodiment of these aspects, the target polypeptide is GPBP, and the assay comprises analyzing the effect(s) of the one or more test compounds on GPBP autophosphorylation. In such an embodiment, an exemplary amount of GPBP for use in the assay is between 50 to 200 ng. In an alternative embodiment, the target polypeptide is selected from the group consisting of an α3 type IV collagen NC1 domain polypeptide comprising the amino acid sequence of SEQ ID NO:52, an MBP polypeptide comprising the amino acid sequence of SEQ ID NO:53, and a prion protein, such as that in SEQ ID NO:54 and the assay is conducted in the presence of a GPBP isoform as recited above, to test for transphosphorylation of the target polypeptide by the protein kinase. In this embodiment, the target polypeptide can comprise a full length α3 type IV collagen NC1 domain polypeptide (including $\alpha3(IV)NC1Asp^9$ SEQ ID NO:57 or $\alpha3(IV)NC1Ala^9$ SEQ ID NO:56), full length MBP, and prion protein, or portions thereof that contain sequences sufficient for phosphorylation by GPBP.

For in vitro phosphorylation assays, detection of phosphorylation can be accomplished by any number of means, including but not limited to using ^{32}P labeled ATP and carrying out autoradiography of a Western blot of the resulting protein products on a reducing or non-reducing gel, or by scintillation counting after a step to separate incorporated from unincorporated label.

Analysis of in vitro phosphorylation may further include identifying the effect of the one or more test compounds on phosphorylation of individual conformational isomers of the target polypeptide. Such identification can be accomplished, for example, by carrying out SDS-PAGE on the reaction products of the phosphorylation reaction, followed by Western blotting, autoradiography and immunodetection of the target protein, as disclosed in WO 02/061430.

Analysis of in vitro phosphorylation may further include identifying the effect of the one or more test compounds on Ser^9 phosphorylation of the α3 type IV collagen NC1 domain, as disclosed in WO 02/061430. Such identification can be accomplished, for example, by comparing the immunoreactive patterns of antibodies specifically reacting with the N terminus of the α3(IV)NC1 (including but not limited to antibodies disclosed in WO 02/061430) and antibodies specifically reacting with Ser(P), such as those commercially available from Sigma Chemical Co. (St. Louis, MO).

The data presented in WO 02/061430 suggest that phosphorylation at Ser^9 exerts a positive control over conformational isomerization of α3(IV)NC1, and efficiently changes the cohort of α3(IV)NC1 conformers produced by a cell. These findings suggest that Ser^9 is one of the structural features that renders the α3(IV)NC1 domain potentially immunogenic, and suggest that, during pathogenesis, an aberrant phosphorylation event on this serine can lead the formation of conformers for which the immune system has not established a tolerance. Thus, determining the effect of test compounds on phosphorylation of the Ser^9 residue of α3 type IV collagen NC1 domain may be important in identifying especially useful candidate compounds for treating autoimmune disorders. Ser^8 in MBP has been shown to be functionally similar to Ser^9 in α3(IV)NC1 conformation and therefore similar tests can be conducted to identify compounds affecting MBP Ser^8 phosphorylation. (See WO 00/50507 and WO 02/061430)

Alternatively, the effects of test compounds on phosphorylation of the target polypeptide can be analyzed in cultured cells. Such a method involves contacting cells that express a target polypeptide selected from the group consisting of an α3 type IV collagen NC1 domain

polypeptide, MBP, and prion protein under conditions to promote phosphorylation, detecting phosphorylation of the target polypeptide; and identifying test compounds that reduce phosphorylation of the target polypeptide relative to phosphorylation of the target polypeptide in the absence of the one or more test compounds. Appropriate cells for use are eukaryotic cells that express the appropriate target protein. Methods of detecting phosphorylation are as described above.

As used herein, the phrase "reduce/reducing phosphorylation" means to lessen the phosphorylation of the target polypeptide relative to phosphorylation of the target polypeptide in the absence of the one or more test compounds. Such "reducing" does not require elimination of phosphorylation, and includes any detectable reduction in phosphorylation. Thus, a test compound that inhibits phosphorylation of the target by, for example, as little as 10-20% would be considered a test compound that reduced phosphorylation. Such a compound may, for example, affect phosphorylation of Ser^9 of the α3(IV) NC1 polypeptide or Ser^8 in MBP, which is shown to exert a powerful control on conformational diversification, and thus to be a strong candidate for an inhibitor of autoimmunity. Alternatively, a test compound may inhibit phosphorylation of target polypeptide by 90%, but have little inhibitory effect on conformational isomerization of the target polypeptide, because reduction affects phosphorylation at sites other than Ser^9 or Ser^8. By performing assays both for phosphorylation inhibition of the target polypeptide, and conformer inhibition of the target polypeptide, it is possible to identify those compounds with the best potential for use as therapeutics for autoimmune disorders.

The above methods can be performed in whole cells or cell extracts expressing recombinant or naturally occurring forms of the polypeptides, in the absence of cells using proteins isolated via any of the methods disclosed herein and optionally including lysosomal extracts, or via any other methods known in the art.

Similarly, inhibition of conformational isomerization of the target polypeptide can be carried out in vitro using isolated components, or can be carried out in cultured cells, although the use of cultured cells is preferred. In a preferred embodiment using cultured cells, identifying compounds that reduce formation of conformational isomers of the target polypeptide comprises:

(a) providing cells that express a target polypeptide selected from the group consisting of α3(IV)NC1 domain, MBP, prion protein, Aβ1-42 and functional equivalents thereof

(b) contacting the cells with one or more GPBP isoforms comprising or consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:2 (for identifying compounds for treating a protein dposit-mediated disorder), SEQ ID NO:4 (for identifying compounds for treating a protein dposit-mediated disorder), SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, or one or more GPBP isoforms selected from the group consisting of 60 kDa GPBP, 44-47 kDa GPBP, and 32 kDa GPBP;

(c) contacting the cells in the presence or absence of one or more test compounds, under conditions that promote conformational isomerization of the target polypeptide catalyzed by the one or more GPBP isoforms in the absence of the one or more test compounds, wherein the contacting of the cells with the one or more test compounds can occur prior to, simultaneous with, or subsequent to contacting the cells with the one or more GPBP isoforms;

(d) detecting conformational isomerization of the target polypeptide; and

iv) identifying test compounds that reduce conformational isomerization of the target polypeptide relative to conformational isomerization of the target polypeptide in the absence of the one or more test compounds.

Appropriate cells for use are eukaryotic cells that express the appropriate target polypeptide. In a preferred embodiment, cell lines stably transfected to express the target polypeptide are used.

In this embodiment, detection of conformational isomers of the target polypeptide, and the effects of the test compounds thereon, generally involve immunodetection using Western blots of non-reducing SDS-PAGE gels containing the polypeptides from the cells. The target polypeptide can be purified via standard techniques (such as using cells transfected with a recombinant target polypeptide that is linked to an epitope tag or other tag to facilitate purification), or cell extracts can be analyzed. In a most preferred embodiment, stable cell lines (such as those disclosed in WO 02/061430) expressing recombinant target polypeptide are used. In some cases, such as for the α3 type IV collagen NC1 domain polypeptide, the target polypeptide is secreted into the medium in a monomeric form, permitting running of serum-free media samples on SDS-PAGE gels and subsequent Western blot analysis and immunodetection. Alternatively, protein extracts from the cells can be made by standard techniques. In a further alternatively, serum free media or otherwise isolated proteins can be used to coat ELISA plates,

followed by similar immunodetection using antibodies that selectively bind to native conformers and either aberrant conformers or all conformers, respectively, and analysis using plate readers.

In a further embodiment, a reduction in conformational isomerization is determined by first subjecting the samples (in vitro reactions or cultured cells) to centrifugation and using the supernatant for limited proteolysis and further analysis of products by either Western blot or mass spectrometry. Alternatively, supernatants can be analyzed by ELISA using monoclonal antibodies that recognize conformational epitopes of the target protein. In this embodiment, it is possible to distinguish between a reduction in conformational isomerization and reduction of random aggregation, since the supernatant is used to analyze conformational isomerization, while the precipitate is used to analyze random aggregation, as described below.

In a preferred embodiment of an in vitro assay for inhibitors of conformational isomerization of the target polypeptide, the method comprises incubating

(a) a target polypeptide selected from the group consisting of α3(IV)NC1 domain, MBP, and prion protein, and functional equivalents thereof

(b) a GPBP isoform comprising an amino acid sequence selected from the group consisting of SEQ ID NO:2 (for identifying compounds for treating a protein dposit-mediated disorder), SEQ ID NO:4 (for identifying compounds for treating a protein dposit-mediated disorder), SEQ ID NO:6,, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28; or a GPBP isoform selected from the group consisting of 60 kDa GPBP, 44-47 kDa GPBP, and 32 kDa GPBP; in the presence or absence of one or more test compounds, under conditions that promote conformational isomerization of the target polypeptide catalyzed by the GPBP isoform in the absence of the one or more test compounds, detecting conformational isomerization of the target polypeptide; and identifying test compounds that reduce conformational isomerization of the target polypeptide relative to conformational isomerization of the target polypeptide in the absence of the one or more test compounds, wherein such compounds are candidate compounds to treat one or more of an autoimmune condition and a protein deposit-mediated disorder.

As used herein, the phrase "reduce/reducing conformational isomerization" means to lessen the formation of conformers of the target polypeptide relative to conformer production

under control conditions. Such “reducing” does not require elimination of conformer formation, and includes any detectable reduction in conformer formation. Furthermore, such “reduction in conformer formation” may entail a reduction in only one, or fewer than all conformational isomers; one can envision that such a reduction in production of specific conformers may be accompanied by an increase in the formation of other conformers. For example, we present evidence in WO 02/061430 that, for the α3(IV) NC1 domain polypeptide, a 27 kDa conformer is the primary product from which the remaining conformers derive. Thus, in a further preferred embodiment, the method comprises identifying those compounds that do not alter the formation of the 27-kDa conformer, but reduce formation of one or more of the other conformers. A preferred method for monitoring this inhibition of specific conformers is to use Mab3 antibody (described in WO 02/061430), which only reacts with the 27-kDa conformer, in parallel with Mab175, which is equally reactive with all α3 type IV collagen NC1 domain conformers.

In a further preferred embodiment of the assays to identify inhibitors of conformational isomerization of the target polypeptide, the target polypeptide is an α3(IV)NC1 domain polypeptide, and analysis of test compound effect on conformer formation of each of wild type α3(IV)NC1 and α3(IV)NC1Asp^9 (SEQ ID NO:57) is carried out in parallel. α3(IV)NC1Asp^9 is modified to replace Ser^9 with Asp^9, an amino acid residue that mimics a permanently phosphorylated residue, which is used herein as an example of an aberrant phosphorylation of α3(IV)NC1, that leads to the production of aberrant conformers. In WO 02/061430, we show that α3(IV)NC1Asp^9 expressing cells produce a larger number of conformers than cells expressing α3(IV)NC1Ser^9. Furthermore α3(IV)NC1Asp^9 cells express a 27-kDa conformer that reacts more strongly with Mab3, as well as with Goodpasture patient autoantibodies, than the 27-kDa conformer produced by α3(IV)NC1Ser^9 expressing cells. It is most preferred to identify compounds that abolish these differences in conformer production between α3(IV)NC1Asp^9 and α3(IV)NC1Ser^9, because this will indicate that the compound inhibits the production of an aberrant 27-kDa conformer from α3(IV)NC1Asp^9, while maintaining appropriate conformer production for α3(IV)NC1Ser^9.

In a further preferred embodiment, identifying compounds for treating an autoimmune disorder further comprises identifying compounds that reduce random aggregation of the target protein. As used herein, "random aggregation" is defined as non-physiological protein aggregation, as opposed to non-random, physiological protein oligomerization. GPBP catalyzes

in vitro oligomerization and prevents random aggregation of protein substrates such as α3(IV)NC1.

While not being limited by a specific mechanism, we propose that the ideal drug candidate for treating autoimmune disorders and/or protein deposit-mediated disorders would inhibit the kinase and chaperonine activity of GPBP, but would not inhibit its chaperone (ie: random aggregate-disrupting) activity (See WO 02/061430), in order to minimize the possibility that inhibition of GPBP activity would lead to increased random aggregate formation. Even more preferably, the ideal drug candidate would, in fact, enhance the chaperone activity of GPBP, to minimize secondary effects derived from undesirable aggregation of conformers.

Both in vitro assays and assays utilizing cultured cells can be used for identifying compounds that reduce random aggregation of the target polypeptide, although in vitro methods are preferred. One embodiment of an in vitro assay comprises:

i) incubating in vitro a target polypeptide selected from the group consisting of α3(IV)NC1, MBP, prion protein, $A\beta_{1-42}$, and functional equivalents thereof, with a GPBP isoform comprising an amino acid sequence selected from the group consisting of SEQ ID NO:2 (for identifying compounds for treating a protein-deposit-mediated disorder), SEQ ID NO:4 (for identifying compounds for treating a protein-deposit-mediated disorder), SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28; or a GPBP isoform selected from the group consisting of 60 kDa GPBP, 44-47 kDa GPBP, and 32 kDa GPBP in the presence or absence of one or more test compounds, under conditions to promote random aggregation of the target polypeptide by the GPBP isoform in the absence of the one or more test compounds; and

ii) identifying test compounds that reduce random aggregation of the target polypeptide by the GPBP isoform relative random aggregation of the target polypeptide by the GPBP isoform in the absence of the one or more test compounds.

Detection of random aggregates, and the effect of test compounds thereon, is preferably carried out by Western blotting of a non-reducing SDS-PAGE gel of the isolated target polypeptide after incubation, and probing with antibodies that recognize the target polypeptide. Preferably, immunodetection is carried out using, in parallel, an antibody that detects a native conformation of the target polypeptide (such as Mab3 which selectively binds to an α3 type IV

collagen NC1 domain polypeptide conformer (WO 02/061430)), and an antibody that detects all target polypeptide conformational isomers (such as Mab175 disclosed in WO 02/061430).

In a further embodiment, detection of random aggregation either in vitro or in cultured cells comprises centrifuging the samples and using the precipitates for direct Western blot analysis or for specific limited proteolysis followed by analysis of proteolytic products by either Western blot analysis or mass spectrometry. In many cases this is a preferred embodiment, as random aggregates of the target protein are generally precipitable, and therefore centrifugation separates random from non-random aggregates.

In a preferred embodiment of the random aggregation assay using cultured cells, cells that express the α3(IV)NC1 domain alone, the entire α3(IV) chain or or type IV collagen containing α3(IV) chain are contacted with the one or more test compounds, and the α3(IV)NC1 domain or collagenase digested α3(IV) chain or type IV collagen produced and secreted by the cells analyzed for α3(IV)NC1 oligomers by Western blot analysis as described in WO 02/061430.

As used herein the phrase "reduce/reducing GPBP induced random aggregation of the target polypeptide" means to decrease the amount of GPBP induced random aggregates of the target polypeptide relative to random aggregation under control conditions. Such "reducing" does not require elimination of random aggregation formation, and includes any detectable reduction in random aggregation formation, including reduction in only a single species of random aggregation in the presence of increased in other species of random aggregates.

In a further embodiment, the method for identifying candidate compounds to treat an autoimmune condition and/or a protein deposit-mediated disorder comprises contacting: (a) a GPBP isoform comprising or consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:2 (for identifying compounds for treating protein deposit-mediated disorders), SEQ ID NO:4 (for identifying compounds for treating protein deposit-mediated disorders), SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28; or a GPBP isoform selected from the group consisting of 60 kDa GPBP, 44-47 kDa GPBP, and 32 kDa GPBP with (b) a target polypeptide selected from the group consisting of α3(IV) NC1 domain, MBP, prion protein, Aβ1-42, and functional equivalents thereof, in the presence of one or more test compounds, under conditions that promote formation of an interaction between the

GPBP isoform and the target polypeptide in the absence of test compounds and identifying test compounds that inhibit the interaction, wherein such compounds are candidate compounds to treat an autoimmune condition and/or a protein deposit-mediated disorder.

Such methods can be performed in whole cells or cell extracts (such as mammalian brain extracts) expressing recombinant or naturally occurring forms of the GPBP isoform and/or the target polypeptide, in the absence of cells using proteins isolated via any of the methods disclosed herein and optionally including lysosomal extracts, or via any other methods known in the art. The interaction between the GPBP isoform and the target polypeptide can be monitored by a variety of methods, including co-immunoprecipitation assays using antibodies directed against the GPBP isoform, the target polypeptide, and/or antibodies directed against expression tags added to recombinant versions of the GPBP isoform and/or the target polypeptide. Alternatively interactions can be monitored by analyzing aggregation kinetics as discussed below.

It should be noted that in each of the above embodiments of methods for detecting candidate compounds for treating an autoimmune condition and/or protein deposit-mediated disorders, conditions can be modified to reduce the pH of the reactions to approximate conditions in cellular compartments to which various GPBP isoforms have been localized. Such reaction conditions may better approximate physiological conditions. For example, a pH in the range of 5 to 5.5 could be used to simulate conditions in the lysosome or 6-6.5 to simulate conditions in the ER/Golgi.

As used herein a "protein deposit-mediated disorder" means a disease mediated by abnormal deposition of a specific protein, including but not limited to Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, prion diseases, and type II diabetes, and autoimmune disorders. The protein deposit may be amyloid matter or para-amyloid matter.

As used herein an "autoimmune condition" is selected from the group consisting of Goodpasture Syndrome, multiple sclerosis, systemic lupus erythematosus, cutaneous lupus erythematosus, pemphigus, pemphigoid and lichen planus.

Modulators of GPBP activity

In another aspect, the present invention provides a method for treating an autoimmune disorder, a tumor, a protein deposit-mediated disorder, and/or for preventing cell apoptosis

comprising modification of the expression or activity of a GPBP isoform comprising or consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:SEQ ID NO:26, SEQ ID NO:28; or a GPBP isoform selected from the group consisting of 60 kDa GPBP, 44-47 kDa GPBP, and 32 kDa GPBP. Modifying the expression or activity of these polypeptides can be accomplished by using inducers or inhibitors of GPBP expression or activity, such as GPBP antibodies, antisense oligonucleotides complimentary to the transcription product of the GPBP gene, small interfering RNAs targeting the transcription product of the GPBP gene, gene or protein therapy using GP or myelin basic protein alternative products, cell therapy using host cells expressing GP or myelin basic protein alternative products, or other techniques known in the art. As used herein, "modification of expression or activity" refers to modifying expression or activity of either the RNA or protein product. Examples of such inducers or inhibitors are discussed below.

As part of the present invention, the inventors have identified further inhibitors of GPBP activity. Thus, in another aspect, the present invention provides an isolated polypeptide consisting of an amino acid sequence according to the general formula X1-SHCIX2-X3, wherein:

X1 is 0-10 amino acids of the sequence ATTAGILATL (SEQ ID NO:41);

X2 is E or Q; and

X2 is 0-10 amino acids of the sequence LMVKREDSWQ (SEQ ID NO:42).

As described below, the inventors have identified the peptide "SHCIE" (SEQ ID NO:39), which is derived from the GPBP sequence disclosed in WO 00/50607, as a key site for self-interaction of GPBP. As such, use of peptides comprising this sequence has been shown to inhibit GPBP kinase activity which makes them useful as therapeutics for a number of indications, as discussed below. A similar sequence (SHCIQ (SEQ ID NO:40)) is present in aggregatable CaM kinase II subunits α, β and δ, whereas it is not present in non-aggregatable CaM kinases I and IV (see below).

X1 and X3 provide optional amino acid sequences from GPBP immediately flanking the core sequence, to provide appropriate secondary structural characteristics to the polypeptide for optimal inhibitory activity.

In a preferred embodiment of this aspect of the invention, the polypeptide consists of a sequence selected from the group consisting of SHCIE (SEQ ID NO:39), SHCIQ (SEQ ID NO:40), ILATLSHCIELMVKR (SEQ ID NO:43), and ILATLSHCIQLMVKR (SEQ ID NO:44).

As described below, the inventors have further identified the peptide EKTAGKPILF (SEQ ID NO:45), present at the carboxy terminus of GPBP, as being a key site for GPBP self-interaction. As such, peptides of 6 or more amino acids derived from this sequence are useful as therapeutics for a number of indications, as discussed below. Thus, in another embodiment, the present invention provides isolated polypeptides consisting of at least 6 amino acids of the sequence EKTAGKPILF (SEQ ID NO:45). In a preferred embodiment, the isolated polypeptide consists of the sequence EKTAGKPILF (SEQ ID NO:45).

The polypeptides according of this aspect of the invention can further be derivatized to provide enhanced half-life, such as by the addition of polyethylene glycol (PEG) or as otherwise known in the art. The polypeptides of the invention may comprise L-amino acids, D-amino acids (which are resistant to L-amino acid-specific proteases in vivo), a combination of D- and L-amino acids, and various "designer" amino acids (e.g., β-methyl amino acids, Cα-methyl amino acids, and Nα-methyl amino acids, etc.) to convey special properties. Synthetic amino acids include ornithine for lysine, and norleucine for leucine or isoleucine.

In addition, the polypeptides can have peptidomimetic bonds, such as ester bonds, to prepare polypeptides with novel properties. For example, a polypeptide may be generated that incorporates a reduced peptide bond, i.e., R_1-CH_2-NH-R_2, where R_1 and R_2 are amino acid residues or sequences. A reduced peptide bond may be introduced as a dipeptide subunit. Such a polypeptide would be resistant to protease activity, and would possess an extended half-live in vivo.

The term "polypeptide" is used in its broadest sense to refer to a sequence of subunit amino acids, amino acid analogs, or peptidomimetics. The subunits are linked by peptide bonds, although the polypeptide can comprise further moieties that are not necessarily linked to the polypeptide by a peptide bond. For example, as discussed above, the polypeptide can further comprise a non-amino acid molecule that contains an aromatic ring.

The polypeptides described herein may be chemically synthesized or recombinantly expressed. Recombinant expression can be accomplished using standard methods in the art, as

disclosed above. Such expression vectors can comprise bacterial or viral expression vectors, and such host cells can be prokaryotic or eukaryotic.

Preferably, the polypeptides for use in the methods of the present invention are chemically synthesized. Synthetic polypeptides, prepared using the well-known techniques of solid phase, liquid phase, or peptide condensation techniques, or any combination thereof, can include natural and unnatural amino acids. Amino acids used for peptide synthesis may be standard Boc (Nα-amino protected Nα-t-butyloxycarbonyl) amino acid resin with standard deprotecting, neutralization, coupling and wash protocols, or standard base-labile Nα-amino protected 9-fluorenylmethoxycarbonyl (Fmoc) amino acids. Both Fmoc and Boc Nα-amino protected amino acids can be obtained from Sigma, Cambridge Research Biochemical, or other chemical companies familiar to those skilled in the art. In addition, the polypeptides can be synthesized with other Nα-protecting groups that are familiar to those skilled in this art.

Solid phase peptide synthesis may be accomplished by techniques familiar to those in the art and provided, such as by using automated synthesizers.

In a further aspect, the present invention provides silencers of GPBP and/or GPBPΔ26 expression, selected from the group consisting of siGPBPΔ26-1 (SEQ ID NO:47), siGPBPΔ26-2 (SEQ ID NO:48), siGPBPΔ26-3 (SEQ ID NO:49), siGPBPΔ26-4 (SEQ ID NO:50), and siGPBP (SEQ ID NO:51). These nucleic acids may be DNA or RNA, and may be single stranded or double stranded (in which case they also include the nucleic acid sequence complementary to the recited sequence, as well be recognized by those of skill in the art), although they are preferablyy RNA and double stranded. When used as DNA they are delivered into the cell in an appropriate vector for intracellular transcription and double stranded RNA synthesis, as is known in the art. As discussed below, each of these silencers was shown to diminish GPBP and/or GPBPΔ26 expression, and thus they are useful for the therapeutic methods of the invention, as discussed below. The silencers can be made by standard methods, such as those disclosed herein.

In a preferred embodiment, the nucleic acids are used in the methods for the invention as double stranded RNAs. Methods for using such double stranded RNAs are as described, for example in US 6,506,559. For example, RNA may be synthesized in vivo or in vitro. Endogenous RNA polymerase of the cell may mediate transcription in vivo, or cloned RNA polymerase can be used for transcription in vivo or in vitro. For transcription from a transgene in vivo or an expression construct, a regulatory region (e.g., promoter, enhancer, silencer, splice

donor and acceptor, polyadenylation) may be used to transcribe the RNA strand (or strands). The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. RNA may be chemically or enzymatically synthesized by manual or automated reactions. The RNA may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). If synthesized chemically or by in vitro enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively, the RNA may be used with no or a minimum of purification to avoid losses due to sample processing. The RNA may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or stabilization of the duplex strands.

In another aspect, the present invention provides pharmaceutical compositions comprising the polypeptide or GPBP silencers of this aspect of the invention or pharmaceutically acceptable salts thereof, and a pharmaceutically acceptable carrier.

These peptides, or pharmaceutical compositions thereof, can be used in methods for treating one or more of autoimmune conditions and a protein deposit-mediated disorder, which comprise providing an amount effective of the polypeptides or GPBP silencers to a patient in need thereof to treat the autoimmune condition and/or a protein deposit-mediated disorder. The terms "autoimmune condition" and " protein deposit-mediated disorder " are as defined above.

As used herein, "treat" or "treating" means accomplishing one or more of the following: (a) reducing the severity of the disorder; (b) limiting or preventing development of symptoms characteristic of the disorder(s) being treated; (c) inhibiting worsening of symptoms characteristic of the disorder(s) being treated; (d) limiting or preventing recurrence of the disorder(s) in patients that have previously had the disorder(s); and (e) limiting or preventing recurrence of symptoms in patients that were previously symptomatic for the disorder(s).

In a further embodiment, the present invention provides methods for inhibiting GPBP activity, comprising administering to a patient in need thereof an amount effective to inhibit GPBP activity of one or more novel polypeptides or silencers according to this aspect of the invention. As used herein, the term "inhibiting" or "inhibit" means to decrease GPBP expression or activity, such as decreasing GPBP kinase activity.

The present invention further provides methods for treating one or more of an autoimmune disorder and a protein deposit-mediated disorder comprising administering to a subject in need thereof an amount effective to treat the disorder of a compound selected from the group consisting of staurosporine, Ca^{2+}CaM, 1-[N,O-bis-(5-Isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN62), and 2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-93), or pharmaceutically acceptable salts thereof. The experimental results below demonstrate that each of these compounds is, either alone or in combination with other compounds, an inhibitor of GPBP activity.

For administration, the polypeptides, nucleic acids, or other compounds disclosed above (hereinafter referred to collectively as "compounds") are ordinarily combined with one or more adjuvants appropriate for the indicated route of administration. The compounds may be mixed with lactose, sucrose, starch powder, cellulose esters of alkanoic acids, stearic acid, talc, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulphuric acids, acacia, gelatin, sodium alginate, polyvinylpyrrolidine, and/or polyvinyl alcohol, and tableted or encapsulated for conventional administration. Alternatively, the compounds of this invention may be dissolved in saline, water, polyethylene glycol, propylene glycol, carboxymethyl cellulose colloidal solutions, ethanol, corn oil, peanut oil, cottonseed oil, sesame oil, tragacanth gum, and/or various buffers. Other adjuvants and modes of administration are well known in the pharmaceutical art. The carrier or diluent may include time delay material, such as glyceryl monostearate or glyceryl distearate alone or with a wax, or other materials well known in the art.

The compounds of the invention can be administered as the sole active pharmaceutical agent, or they can be used in combination with one or more other compounds useful for carrying out the methods of the invention. When administered as a combination, the therapeutic agents can be formulated as separate compositions that are given at the same time or different times, or the therapeutic agents can be given as a single composition.

The compounds may be made up in a solid form (including granules, powders or suppositories) or in a liquid form (*e.g.*, solutions, suspensions, or emulsions). The compounds of the invention may be applied in a variety of solutions and may be subjected to conventional

pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc.

The compounds of the invention may be administered orally, topically, parenterally, by inhalation or spray or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and vehicles. The term parenteral as used herein includes percutaneous, subcutaneous, intravascular (e.g., intravenous), intramuscular, or intrathecal injection or infusion techniques and the like. In addition, there is provided a pharmaceutical formulation comprising a compound of the invention and a pharmaceutically acceptable carrier. One or more compounds of the invention may be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants, and if desired other active ingredients. The pharmaceutical compositions containing compounds of the invention may be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

Compositions intended for oral use may be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions may contain one or more agents selected from the group consisting of sweetening agents, flavoring agents, coloring agents and preservative agents in order to provide palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients that are suitable for the manufacture of tablets. These excipients may be for example, inert diluents, such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example starch, gelatin or acacia, and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets may be uncoated or they may be coated by known techniques. In some cases such coatings may be prepared by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monosterate or glyceryl distearate may be employed.

Formulations for oral use may also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium

phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil.

Aqueous suspensions contain the active materials in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydropropyl-methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents may be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions may also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.

Oily suspensions may be formulated by suspending the active ingredients in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions may contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents and flavoring agents may be added to provide palatable oral preparations. These compositions may be preserved by the addition of an anti-oxidant such as ascorbic acid.

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents or suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavoring and coloring agents, may also be present.

Pharmaceutical compositions of the invention may also be in the form of oil-in-water emulsions. The oily phase may be a vegetable oil or a mineral oil or mixtures of these. Suitable emulsifying agents may be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol, anhydrides, for example sorbitan monooleate, and

condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsions may also contain sweetening and flavoring agents.

Syrups and elixirs may be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol, glucose or sucrose. Such formulations may also contain a demulcent, a preservative and flavoring and coloring agents. The pharmaceutical compositions may be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents that have been mentioned above. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono-or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The compounds and pharmaceutical compositions of the present invention may also be administered in the form of suppositories, e.g., for rectal administration of the drug. These compositions can be prepared by mixing the drug with a suitable non-irritating excipient that is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such materials include cocoa butter and polyethylene glycols.

Compounds and pharmaceutical compositions of the present invention may be administered parenterally in a sterile medium. The drug, depending on the vehicle and concentration used, can either be suspended or dissolved in the vehicle. Advantageously, adjuvants such as local anesthetics, preservatives and buffering agents can be dissolved in the vehicle.

Dosage levels of the order of from about 0.01 mg to about 50 mg per kilogram of body weight per day, and more preferably between 0.1 mg to about 50 mg per kilogram of body weight per day, are useful in the treatment of the above-indicated conditions. The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration.

Dosage unit forms will generally contain between from about 1 mg to about 500 mg of an active ingredient.

Pharmaceutical compositions containing the compounds described herein are administered to an individual in need thereof. In a preferred embodiment, the subject is a mammal; in a more preferred embodiment, the subject is a human. In therapeutic applications, compositions are administered in an amount sufficient to carry out the methods of the invention. Amounts effective for these uses depend on factors including, but not limited to, the nature of the compound (specific activity, etc.), the route of administration, the stage and severity of the disorder, the weight and general state of health of the subject, and the judgment of the prescribing physician. The active compounds are effective over a wide dosage range. However, it will be understood that the amount of the compound actually administered will be determined by a physician, in the light of the above relevant circumstances. Therefore, the above dosage ranges are not intended to limit the scope of the invention in any way.

The present invention may be better understood with reference to the accompanying examples that are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined by the claims appended hereto.

Examples

Synthetic oligonucleotides. The following oligonucleotides and others used for cDNA sequencing were synthesized by Amershan Biosiences o Roche:

ON-hmbGPBP-5c, 5´-CCTCCGAGCCCGACGAGTTC-3´ (SEQ ID NO:58)

ON-dinb1, 5´- GACCGAAAGGGGCACGCAAC-3´; (SEQ ID NO:59)

ON-GPBPΔ102, 5´-AAAAAGAATTCGCATCGAGGGGGCTAAGTTCGG-3´; (SEQ ID NO:60)

ON-GPBPΔ174, 5´-AAAAAGAATTCGACGGCTGGAAGGGTAGGCT-3´; (SEQ ID NO:61)

ON-GPBPΔ246, 5´-AAAAAGAATTCTGTCAGGCGCGGCGGCGGCGC-3´; (SEQ ID NO:62)

ON-GPBPΔ315, 5´-GACGAATTCCCATCCCCCGACCCTTCACCC-3´; (SEQ ID NO:63)

ON-GPBPΔ369, 5´-AAAAAGAATTCGGAGCGGGGGCCGGTCTCCTGC-3´; (SEQ ID NO:64)

ON-pU1, 5´-ACGACTCACTATAGGGAGAC-3´; (SEQ ID NO:65)

ON-pcDNAc, 5′-CTCTAGCATTTAGGTGACAC-3′; (SEQ ID NO:66)

ON-GPBPMet$_{mut}$ 5′-GGTTGTCGAGCCTCCGGATCGGATAATCAGAGC-3'; (SEQ ID NO:67)

ON-PrP-F3, 5'-GAGAATTCAGCAGTCATTATGGCGAACCTT-3'; (SEQ ID NO:68)

ON- PrP-R1, 5'-GAACTCGAGCCTTCCTCATCCCACTATCAGG-3'; (SEQ ID NO:69)

ON-E/K-PrP-F6, 5'- TATCACCCAGTACAAGAGGGAATCT-3'; (SEQ ID NO:70)

ON-E/K-PrP-R6, 5'- AGATTCCCTCTTGTACTGGGTGATA-3'; (SEQ ID NO:71)

ON-E168R-F1, 5'-CCCATGGATAGGTACAGCAACC-3'; (SEQ ID NO:72)

ON-E168R-R1, 5'-GGTTGCTGTACCTATCCATGGG-3'; (SEQ ID NO:73)

ON-Q172R-F1, 5'-GAGTACAGCAACAGGAACAACTTTG-3'; (SEQ ID NO:74)

ON-Q172R-R1, 5'-CAAAGTTGTTCCTGTTGCTGTACTC-3'; (SEQ ID NO:75)

ON-R220A-F1, 5'-CAGTACGAGGCGGAATCTCAGG-3'; (SEQ ID NO:76)

ON-R220A-R1, 5'-CCTGAGATTCCGCCTCGTACTG-3'; (SEQ ID NO:77)

ON-R228A-F1, 5'-TATTACCAGGCAGGATCGAGCAT-3'; (SEQ ID NO:78)

ON-R228A-R1, 5'-ATGCTCGATCCTGCCTGGTAATA (SEQ ID NO:79)

cDNA cloning and plasmid constructs for deletion mutants. To generate the cDNA for the different GPBP deletion mutants, we performed PCR on pc-n4' using one of the following synthetic oligonucleotides ON-GPBPΔ102, ON-GPBPΔ174, ON-GPBPΔ246, ON-GPBPΔ315, ON-GPBPΔ369 and ON-pcDNAc. The resulting cDNAs were individually cloned in EcoRI of pc-DNA3 (Invitrogen) to generate the pc-n4'Δ series. To obtain YFP-Flag-n4' and YFP-n4'Δ102, the cDNAs in pc-Flag-n4'and pc-n4'Δ102 were EcoRI excised and cloned in-frame into pEYFP-C1 (Clontech).

The cloning identification and characterization of cDNA for bovine and mouse GPBP and GPBPΔ26 has been reported (WO 00/50607 and WO 02/061430). The 5' UTR region for rat GPBP mRNA was obtained by standard reverse transcriptase-coupled-PCR using ON-hmbGPBP-5c and ON-dinb1 and total RNA was extracted from cultured rat astrocytes provided by C. Guerri at FVIB, and subsequent nucleotide sequencing of PCR product.

The pc-n4'Met$_{mut}$ construct was obtained by Transformer^{TM}Site-Directed Mutagenesis (Clontech) using pc-n4' and ON-GPBPMet$_{mut}$ following manufacturer's instructions.

We used human DNA extracted from blood and ON-PrP-F3 and ON-PrP-R1 to obtain a DNA that was subsequently cloned in EcoRI and XhoI of pc-DNA3 (Invitrogen) to produce pc-PrP. To produce the derived mutants we used a double-PCR approach using complementary oligonucleotides (ON-E/K-PrP-F6/ON-E/K-PrP-R6; ON-E168R-F1/ ON-E168R-R1;ON-Q172R-F1/ON-Q172R-R1;ON-R220A-F1/ON-R220A-R1;ON-R228A-F1/ON-R228A-R1) that introduce the desired mutation and ON-pcDNAc or ON-pU1, and pc-PrP as a template. The resulting DNAs were similarly cloned in pc-DNA3.

SimRNA production. Silencers were generated using pSilencer 2.1-U6 hygro plasmid (Ambion) following manufacturers recommendations. The oligonucleotide pairs used were:

SiGPBP/Δ26-1:
5'GATCCCACTACATTCATGGGTGGCATTCAAGAGATGCCACCCATGAATGTAGTTTTTTTGGAAA-3' (SEQ ID NO:80) and

5'AGCTTTTCCAAAAAAACTACATTCATGGGTGGCATCTCTTGAATGCCACCCATGAATGTAGTGG-3' (SEQ ID NO:81).

SiGPBP/Δ26-2:
5'GATCCCACAGAGTATGGCTGCAGAGTTCAAGAGACTCTGCAGCCATACTCTGTTTTTTTGGAAA-3' (SEQ ID NO:82) and

5'AGCTTTTCCAAAAAAACAGAGTATGGCTGCAGAGTCTCTTGAACTCTGCAGCCATACTCTGTGG-3' (SEQ ID NO:83);

SiGPBP/Δ26-3:
5'GATCCCGTACTTTGATGCCTGTGCTTTCAAGAGAAGCACAGGCATCAAAGTACTTTTTTGGAAA-3' (SEQ ID NO:84) and
5'AGCTTTTCCAAAAAAGTACTTTGATGCCTGTGCTTCTCTTGAAAGCACAGGCATCAAAGTACGG-3' (SEQ ID NO:85);

SiGPBP/Δ26-4:

5'GATCCCAGGCGTCACAGGACATGAATTCAAGAGATTCATGTCCTGTGACGCCTTTT TTTGGAAA-3' (SEQ ID NO:86) and

5'AGCTTTTCCAAAAAAAGGCGTCACAGGACATGAATCTCTTGAATTCATGTCCTGTG ACGCCTGG-3' (SEQ ID NO:87);

SiGPBP:

5'GATCCCGCCCTATAGTCGCTCTTCCTTCAAGAGAGGAAGAGCGACTATAGGGCTTT TTTGGAAA-3' (SEQ ID NO:88) and 5'AGCTTTTCCAAAAAAGCCCTATAGTCGCTCTTCCTCTCTTGAAGGAAGAGCGACTA TAGGGCGG-3' (SEQ ID NO:89).

GPBP Expression in yeast and purification of recombinant protein. Recombinant FLAG-tagged human GPBP was essentially prepared as indicated in Raya, A., Revert, F., Navarro, S., and Saùs J (1999) J. Biol. Chem.274, 12642-12649 . For light scattering purposes FLAG-affinity purified GPBP was further purified by FPLC on a Resource-Q column (Amersham Bioscience) equilibrated with 20mM Tris HCl pH 8 and eluted in a linear gradient of NaCl 0-1M established in the same buffer and the peak containing the material which eluted at ~0.6 M NaCl was aliquot and stored at –80°C until use.

Recombinant protein expression in cultured cells. The pcDNA3-based contructs containing the cDNA encoding the different human proteins of interest were used to transfect human 293 cells using standard calcium phosphate procedures in ProFection Mammalian Transfection System (Promega). 24-48 h after transfection cell lysates were used for Western blot, precipitation or immunoprecipitation studies. Cells expressing GPBP or derived deletion mutants were collected on ice with 50 mM Tris HCl pH 7.4, 0.05% Triton X-100, 1 mM PMSF and 5 μg/ml leupeptin, disrupted by vortex and insoluble material discarded by centrifugation at 14.000 rpm in Eppendorf at 4° C for 10 min and supernatant used for Western blot analysis.

For other purposes after transfection and prior analysis cells were incubated with GPBP modulators or lysosomal inhibitors.

Recombinant protein expression in a cell-free system. Approximately 1 μg of the pcDNA3-based construct was expressed in a coupled transcription-translation system (Promega) following manufacturer´s recommendation and using ^{35}S-Methionine. The mixtures were analyzed by SDS-PAGE and a standard procedure for fluorography.

Subcellular fractioning and related studies. Rat liver subcellular fractionation was essentially performed as indicated in Aniento, F., Roche, E., Cuervo, A.M., Knecht, E. (1993). J. Biol. Chem. 268, 10463-10470. For some purposes lysosomal fractions (freshly prepared entire lysosomes) were dispersed in pure water and subjected to ten consecutive cycles of freezing and thawing to alter lysosomal membrane integrity (broken lysosomes). For other purposes, lysosomal fractions were similarly disrupted in the presence of protease inhibitors (PMSF 2 mM, leupeptin 0.2 mM and EDTA 2mM) and subsequently centrifuged 130,000 xg for 10 min at room temp to separate the soluble lysosomal fraction (also called here lysosomal extract) from the non-soluble fraction which, after rinse with 0.3 M saccharose in 10 mM MOPS pH 7.2, was used as the lysosomal membrane fraction.

To determine whether the GPBP immunoreactive polypeptides in lysosomal, microsomal and mitochondrial fractions represented cytoplasmic components non-specifically bound to these organelles we subjected each individual fraction to five consecutive washes with 0.3 M saccharose in 10 mM MOPS pH 7.2. Similar amounts of individual fractions representing each wash were analyzed by Western blot using Mab6. For similar purposes, mitochondrial and lysosomal fractions were treated with trypsine at different concentrations for 1 h at room temperature, digestion stopped by adding soybean trypsin inhibitor and samples similarly analyzed. In these cases, Western blot was performed in parallel with Mab6 and either anti-catepsin D antibodies for lysosomal samples or anti-carbamyl phosphate synthetase for mitochondrial fractions as degradation control of an integral component.

For still other purposes, entire or broken lysosomes (50 μg) were incubated at 30° C with 25 mM β-glycerol phosphate pH 7, 0.5 mM EGTA, 8 mM Mg Cl_2, 5 mM $MnCl_2$, 1 mM DTT, y 0.132 μM [γ^{32}P]-ATP, maintaining saccharose concentration to 0.25 M in a final volume of 50 μl. The time of incubation was between 0 and 60 min and the phosphate transfer reactions stopped by adding SDS-PAGE sample buffer and heating at 95°C.

Fluorescence microscopy studies. In a typical assay 20.000 cells were seeded on glass slides and after 12 h the cells were rinsed with PBS (phosphate buffered saline) and fixed with

methanol/acetone (50:50) for 5 min at –20ºC. Cells were brought to room temperature by rinsing with PBS and used for indirect immunofluorescence. Briefly, cells were blocked for 45 min with PBS 3% BSA and then subsequently incubated for periods of 45 min with the corresponding primary and secondary antibodies. Finally, the slides were mounted for observation. A Zeiss Axioskop 2 microscope was used for standard fluorescence microscopy and an ACAS 570 interactive laser cytometer using a pinhole size of 225 mm corresponding to a 0.99 mm slice for confocal microscopy.

For other purposes, Cos-1 and HeLa cells were grown in 22 mm glass coverslips and transient transfections were performed 24-36 hours after seeding using SuperFect (Qiagen) or Fugene (Roche) transfection reagents. 24-48 hours after transfection, cells were washed with HBSS (Hanks buffered salt solution) containing 5 mM glucose and 10 mM Hepes, pH 7.4. Coverslips were transferred to a microscopy chamber (Attofluor, Molecular Probes, The Netherlands) and cell fluorescence was imaged with an epifluorescence inverted microscope (DMIRE-2, Leica Microsystems, Germany) equipped with an oil immersion 40x objective (NA 1.25). Fluorescence was excited at 475 nm (YFP) using a monocromator (Hamamatsu Photonics, Japan) and emitted light collected by a CCD camera (Orca-ER, Hamamatsu Photonics, Japan). The emission filter (Omega Optical, Brattleboro, VT, USA) was 535± 13 nm and the beam splitter was 445DRLP. Images were acquired and analyzed using the Aquacosmos software (Hamamatsu Photonics, Japan).

Animal studies. NZW, male or female, 4-6 month-old were injected intraperitoneally either with 1 μg/g of body weight of DAB-Am-4 and/or with 20 μg/g of body weight of the Q_{2L} or Q_{2D} peptide. These products were administered in a volume of 500 μl of steril saline solution 3 times per week, at alternate days, during 12 consecutive weeks. Age-matched uninjected mice were used as controls. At the end of the experiment, mice were sacrificed and kidneys were fixed in 10% paraformaldehide and processed for pathological studies. Similar DAB-Am-4 treatment studies were performed on C57BL/6 animals for genetic background control.

Light-scattering studies. A 0.7 μM solution of bovine recombinant PrP^C (Prionics) in 20 mM Mes pH 6.5 buffer supplemented with 20 mM NaCl and 1 mM sodium citrate was placed in the measurement cell. After 10 min FPLC-purified human recombinant GPBP was added from a stock solution in TBS (Tris-buffered saline, 50 mM Tris-HCl pH 8, 150 mM NaCl) to rich a final concentration of 0.19 μM. In a second type of experiment the protein initially placed

in the measurement cell was GPBP and PrP^C was the added protein. For other purposes, GPBP solution was placed in the measurement cell and 10 min after inhibitory Q_{2L} or non-active Q_{2Lr} (100 μM) or Q_{2D} (20 μM) were added, incubation continued for an additional 5-10 min period and PrP^C added. Light scattering at 90° was recorded on a JASCO FP6500 spectrofluorimeter at 500 nm as a function of time.

SDS-PAGE, Western and far Western studies. These studies were essentially performed as indicated in Raya , A., Revert, F., Navarro, S., and Saus J (1999) J. Biol. Chem.274, 12642-12649 and Raya, A. et al., (2000) J. Biol. Chem. 275, 40392-40399.

Yeast two-hybrid studies. Yeast two hybrid-studies to map interactive motifs for GPBP self-aggregation were performed essentially as described in Raya, A. et al., (2000) J. Biol. Chem. 275, 40392-40399 using different deletion mutants for GPBP obtained by standard DNA recombinant techniques.

Precipitation and immunoprecipitation studies. After transfection or modulator treatment, cells were washed once with ice-cold PBS, lysed with 100-300 μl of lysis buffer (20 mM Tris-HCl pH 8, 100 mM NaCl, 0.5% NP-40 0.5%, sodium deoxycholate, 1 mM PMSF and leupeptin 10 μg/ml) and protein concentration estimated using Bio-Rad protein assay and bovine serum albumin as standard. For precipitation studies, equal amounts of protein were brought to 50 μl with lysis buffer and centrifuged at 16.000 x g for 15 min at 4° C. Supernatants and pellets were analyzed by Western blot. For immunoprecipitation studies lystes were pre-cleared at 500 x g for 5 min at 4° C before protein quantification and equal amounts of protein were brought to 250 μl with lysis buffer. 5 volumes were diluted with TBS and incubated with anti-FLAG M2-Agarose Affinity Gel (Sigma) for 1 h at 4° C with gentle agitation; beds were washed three times with TBS and used for Western blot analysis using biotin-labeled antibodies.

Cell cultures with GPBP modulators. One day after transfection (for PrP-expressing cells) or after seeding (for α3(IV)NC1-expressing cells), culture media were replaced with media (PrP-expressing cells) or serum-free media (α3(IV)NC1-expressing cells) containing GPBP modulators and cultures were extended for an additional 24 h (PrP-expressing cells) or 24-48 h (α3(IV)NC1-expressing cells). Cell lysates from PrP expressing cells were used for Western blot, precipitation and immunoprecipitation studies, and culture media from α3(IV)NC1 expressing cells for Western blot analysis. For α3(IV)NC1-expressing cells, synthetic peptides were used at

100-200 μM and organic compounds at 5-50 μM. For PrP-expressing cells, Q_{2D} was used at 1-10 μM , DAB-Am-4 at 1-5 μM and DAB-Am-32 at 0.25-1 μM.

***In vitro* phosphorylation.** These studies were essentially performed as described in Raya , A., Revert, F., Navarro, S., and Saus J (1999) J. Biol. Chem. 274, 12642-12649 . Where indicated GPBP modulators were used at 200 μM in a 10 min autophosphorylation reaction. Further autophosphorylation studies were performed using DAB-Am-4 and Q_{2D} and we have determined that similar activation and inhibition effects were obtained using decreasing concentrations up to 10 μM.

Histochemical and immunohistochemical on paraffin-embedded tissues. Immunohistochemical studies were essentially performed as indicated in Raya, A., Revert, F., Navarro, S., and Saus J (1999) J. Biol. Chem. 274, 12642-12649 and Raya, A. et al., (2000) J. Biol. Chem. 275, 40392-40399. Hematoxylin/eosin and trichromic Mason staining on mice kidney samples were performed following standard procedures.

Antibody production. The production of chicken polyclonal antibodies against GPBPpep1 recognizing GPBP and monoclonal antibodies against GST-GPBP recognizing GPBP/GPBPΔ26 (Mab14) have been previously described in Raya, A., Revert, F., Navarro, S., and Saus J (1999) J. Biol. Chem. 274, 12642-12649 and Raya, A. et al., (2000) J. Biol. Chem. 275, 40392-40399. Similar procedures were used for production of chicken polyclonal antibodies against GPBPpep2 recognizing non-canonical sequence of GPBP/GPBPΔ26 and monoclonal antibodies against GPBPpep1 only reacting GPBP (Mab6). For immunofluorescence and immunohistochemistry studies we used polyclonal antibodies whereas monoclonals were used for Western and far Western blot studies. The production and characterization of monoclonal antibodies against α3(IV)NC1 domain was previously reported (WO 02/061430). For some purposes antibody biotinylation was performed as described in AntibodyArray™ Instruction Manual from Hypromatrix.

Cell lines. The human cells lines used were HEK293 (ATCC), hTERT-RPE1 and hTERTBJ1 (Clontech). The cell line used for α3(IV)NC1 expression was obtained by stably transfecting HEK293 cells and its production has been previously reported (WO 02/061430).

Other products: Synthetic peptides GPBPpep1, Ac-PYSRSSSMSSIDLVSASDDVHRFSSQ-NH2 (**SEQ ID NO:46**) and GPBPpep2, Ac-PRSARCQARRRRGGRTSS-NH2 (**SEQ ID NO:36**) were from Genosys. Synthetic peptides Q_4

(Ac- EKTAGKPILF-OH) **(SEQ ID NO:45)**, Q_{2LI}(Ac-ILATLSHCIELMVKR-NH2) **(SEQ ID NO:43)**, Q_{2L}(Ac-LATLSHCIELMVKR-NH2) **(SEQ ID NO:90)** Q_{2Lr} (Ac-VLMASLETLCRIHKI-NH2) **(SEQ ID NO:92)**, Q_{2DI} (Ac-ILATLSHCIELMVKR-NH2) **(SEQ ID NO:43)** and Q_{2D} (Ac-LATLSHCIELMVKR-NH2) **(SEQ ID NO:90)** were synthesized at the FVIB. Initial in vitro and ex vivo studies were performed using Q_{2LI} and Q_{2DI} however further synthesis and uses were performed in absence of first isoleucine and we synthesized Q_{2L} and Q_{2D} peptides that show similar activity both in vitro and ex vivo but were more soluble and used for animal studies. Antibodies for co-localization were anti-catepsin D from Santa Cruz Biotechnology; anti-Golgin-91 (CDF4) and anti-human E2 subunit of pyruvate dehydrogenase from Molecular Probes. Anti-GAPDH and anti-carbamoyl phosphate synthetase were kindly provided by E. Knecht and J. Cervera at FVIB. GPpep1bov (Ac-KGKPGDTGPPAAGAVMRGFVFT-NH2) **(SEQ ID NO:93)** was synthesized by DiverDrugs and antibodies specific provided by Billy G. Hudson. $A\beta_{1-42}$ and FLAG peptides and the corresponding specific antibodies were from Sigma. All the conjugates used except anti-mouse Ig peroxidase (Promega) were from Sigma. Recombinant bovine PrP was from Prionics. PrP-specific antibodies were from Chemicon (clone 3F4) or from Santa Cruz Biotechnology (C-20). Rat cerebellar neuronal extracts were prepared essentially as described in Miñana MD, Montoliu C, Llansola M, Grisolía S, Felipo V. (1998) Neuropharmacology 137; 847-857 were provided by V. Felipo at FVIB. SiGFP, an mRNA silencer for green fluorescence protein was from Ambion.

RESULTS

Identification of a 91-kDa isoform of GPBP (91 kDa GPBP) as non canonical mRNA translation start site product. We have made the observation that the 5' untranslatable region (5'UTR) of the mRNA of human GPBP contains an upstream open reading frame (ORF) of 130 residues with an in-frame stop codon at the beginning (See WO 00/50607). In vitro or ex vivo translation of the n4' mRNA (n4') resulted in the expression of two molecular species, one consistent with canonical translation at iMet displaying a molecular mass of ~77-kDa (77 kDa GPBP) and other with an apparent higher molecular mass (~91-kDa). To investigate the nature of the 91-kDa molecular species (91 kDa GPBP), a cDNA representing an mRNA with no 5'UTR was obtained and similarly expressed. The expression of this mRNA mutant resulted in a single protein of ~77-kDa (77 kDa GPBP), indicating that the existence of 91-kDa GPBP

depends on non-canonical translation of the ORF at the 5'UTR. A cDNA representing a Met to Gly mRNA mutant for translation initiation expressed only the 91-kDa molecular species (**Fig. 1**), indicating that the 91-kDa GPBP is expressed from a non-canonical translation start site located 5' from the codon encoding the canonical Met initiation codon. Similar 91 kDa GPBP isoforms were shown to be present in mouse and rat cells, and are predicted to be expressed from GPBP mRNA in bovine cells.

The 91-kDa GPBP isoform results from previously unrecognized mRNA translation mechanism. To explore the mechanism underlying expression of 91-kDa GPBP, we generated mutants representing truncated versions of the mRNA at the 5'UTR and performed recombinant expression in a cell-free system (in vitro) or in cultured human cells (ex vivo) (**Fig. 2**).

Whereas all the deletion mutants expressed the canonical polypeptide of 77-kDa GPBP, 91-kDa GPBP was only expressed from the complete mRNA (n4') and from a mutant which is devoid of the 5' 102 nucleotides (**Δ102**). Additional 5' deletions failed to abolish non-canonical translation initiation and caused a gradual reduction in the size of the non-canonical product (**Fig. 2**), suggesting that there are multiple non-canonical translation start sites displaying 5' to 3' hierarchy.

The relative expression of the two polypeptides in cell-free system (in vitro) sharply contrasted with the levels of these two polypeptides when the mRNA was expressed inside the cell, in which case 77-kDa GPBP isoform was significantly more abundant.

In cells and tissues, the expression of GPBP mainly depends on the non-conventional translation of the corresponding mRNA. In a first attempt to investigate the significance of our findings we compared recombinant and endogenous expression of GPBP in cultured human 293 cells (**Fig. 3**). As expected monoclonal antibodies specifically recognizing GPBP (Mab6) reacted with the two recombinant molecular species being expressed from cDNA in cell extracts deriving from transfected cells (91 kDa GPBP and 77 kDa GPBP) (lane 1). Cell extracts derived from non-transfected cells expressed several reactive polypeptides (lane 2), one co-migrating with recombinant 91-kDa GPBP and other polypeptides of higher and lower molecular mass (120-, 47- and 32-kDa), none of which displayed the molecular mass of the canonical recombinant polypeptide (compare lanes 1 and 2). In some studies the presence of an additional 60-kDa polypeptide also was evident. The specificity of the multiple reactivity displayed by

Mab6 was confirmed by full inhibiting antibody binding in the presence of GPBPpep1, a synthetic peptide representing the GPBP exclusive 26-residues used in Mab6 production (lane 3). These findings suggest that at the steady state of the cell canonical translation product is virtually absent whereas non-canonical 91-kDa GPBP product is comparatively more abundant. Furthermore, we identified an additional major GPBP isoforms of 120-kDa along with minor lower molecular mass GPBP isoforms of 60-kDa, 47-kDa and 32-kDa.

To further investigate the nature of the immunoreactive polypeptides, we generated a number of GPBP mRNA silencers and assayed their capacity to inhibit endogenous expression of GPBP related polypeptides (**Fig. 4**). All individual mRNA silencers displaying the capacity to inhibit recombinant GPBP expression (not shown) also negatively impacted endogenous expression of 91 kDa GPBP and 120-kDa GPBP. The consequences on expression of GPBP polypeptides of lower molecular mass varied substantially between silencers. Thus, silencers that were more efficient reducing 91 kDa GPBP and 120-kDa GPBP promoted the expression of 60 kDa GPBP and reduced in an opposite but coordinated manner the expression of 47-kDa GPBP. All these data suggest that the expression of 91 kDa GPBP and 120-kDa GPBP depends more on mRNA translation than the expression of GPBP isoforms of lower molecular mass, which depend on a complex degradation program operating on the primary products and involving a positive feedback of the primary products in the proteolytic step from 60- to 47-kDa. Our findings support that all the polypeptides reactive with Mab6 indeed are GPBP related products, and suggest that at the cellular steady state GPBP expression depends more on non-canonical translation than on canonical translation of the mRNA.

We have extensively studied the expression of GPBP in multiple human tissue extracts and found an expression pattern that in general was similar to that found with cultured cells with the exception of a human striated muscle sample in which case we identified a minor reactive polypeptide of 77-kDa. In this case it remained unclear whether the 77-kDa polypeptide represented a canonical translation event or represented a proteolytical intermediate deriving from non-canonical 91-kDa GPBP (see below). Although the structural relationship between the 91-kDa and polypeptides of higher and lower molecular mass remains to be determined, the evidence suggest that non-canonical translation is more relevant in vivo than canonical. Immunochemical studies performed on paraffin embedded human tissues revealed that the immunostaining patterns obtained using antibodies that recognize canonical and non-canonical

GPBP isoforms are virtually identical to those obtained with antibodies only reacting with non-canonical GPBP products.

In cultured cells, GPBP shows multiple subcellular localization including a prominent presence at the endosomal/lysosomal compartment. Immunohistochemical studies support that non-canonical GPBP isoforms display a broad subcellular localization including extracellular matrix, plasma membrane, cytosol (homogenous, fibillar and granular) and nucleus (WO 00/50607). An analysis for prediction of subcellular localization supports the multiple localization for non-canonical *versus* canonical products (see below), despite the fact that many of these destinies are non-compatible using conventional protein sorting routes. Consequently, we have explored GPBP subcellular localization using conventional immunofluorescence and confocal microscopy in cultured human cells. Indirect immunofluorescence studies on human RPE and BJ1 cells respectively representing epithelial and fibroblastic type of cells immortalized by telomerase (Clontech) revealed two principal cellular expression patterns for GPBP. Most of the cells express GPBP at the cytosol in a diffuse and fibrillar manner, with a remarkable expression of the protein at the nuclear membrane and perhaps presence of the protein in the nuclear environment. A limited number of cells show abundant GPBP expression at granular structures that distribute in the perinuclear region. The percentage of cells expressing the intense granular pattern varied between 10-30% and was more abundant in BJ1 than in RPE cells.

We have explored the cells in which GPBP is over expressed at defined granules to identify their subcellular nature. We performed conventional double indirect immunofluorescence and confocal microscopy using validated immunological probes for secondary lysosomes, Golgi apparatus or mitochondria. These studies revealed that GPBP shows a preferential localization at the lysosomes and a more limited but significant presence in Golgi apparatus and mitochondria. We have also performed fluorescence studies directed to address the intracellular distribution of proteins representing canonical or non-canonical GPBP primary products fused to yellow fluorescence protein. These studies revealed a major granular distribution for the non-canonical GPBP whereas the canonical GPBP appeared to be mainly diffuse cytosolic.

All these results suggest that the endosomal/lysosomal compartment is a principal subcellular destiny for non-canonical GPBP.

The 91-kDa GPBP is the precursor of multiple related polypeptides including lysosomal 44-47-kDa isoforms. To investigate subcellular localization of GPBP in tissues we have used rat liver, a validated and reliable model for cell subfractioning. From the corresponding homogenates we prepared cytosolic, mitochondrial, microsomal and lysosomal fractions and assessed the presence of GPBP by Western blot (**Fig. 5**). The antibodies reacted with multiple polypeptides, which displayed 120-, 91-, 77-, 60-, 44-47, and 32-kDa. The distribution of reactive polypeptides among cellular fractions greatly varied. The polypeptides of higher molecular mass, (120-, 91-, and 77-kDa) were found preferentially in microsomes, the polypeptide of 60-kDa was mainly found in cytosol and mitochondria with traces in microsomes, whereas 44-47-kDa polypeptides were essentially lysosomal. Finally, the 32-kDa polypeptide was the most widely distributed being found in every fraction, followed by the 60-kDa polypeptide that was found in all fractions except in lysosomes. Extensive washing or trypsin treatment of either mitochondrial or lysosomal fractions resulted in no significant reduction in the content of immunoreactive peptides, suggesting that these polypeptides are integral components of these cell compartments.

All our findings suggest that the primary products of GPBP mRNA translation are subjected to a complex intracellular processing coupled to subcellular localization. Immunofluorescence studies suggest that the endosomal/lysosomal compartment is among the most prominent destinations for GPBP. This compartment is also of major interest in antibody-mediated autoimmune pathogenesis and in tissue degeneration, since is actively implicated in the production of both non-tolerized peptides and in protein deposition. To investigate the presence of GPBP in this compartment, we isolated lysosomes from the liver of untreated or leupeptin-treated rats, and the presence of GPBP was investigated by Western blot analysis using specific monoclonal antibodies (**Fig. 6**). Lysosomes from untreated animals contained major reactive polypeptides of 44-47-kDa and a minor polypeptide of 32-kDa. Treatment with leupeptin substantially changed the immunoreactive pattern and thus polypeptides of higher molecular mass (91- and 60-kDa), virtually undetectable in untreated lysosomes were the most abundant whereas the 44-47-kDa polypeptides significantly diminished. Although in most of the cases leupeptin treatment did not change the level of 32-kDa there were examples in which we found increased levels of this polypeptide, and in some other cases treatment was associated with

detection of 77-kDa and 120-kDa polypeptides. These data suggest that the 44-47-kDa polypeptides are integral components of the lysosome that derive from limited leupeptin-sensitive proteolysis of a 91-kDa precursor through a major intermediate of 60-kDa. Furthermore, the non-reduced or moderated augmented expression of 32-kDa polypeptide associated with leupeptin treatment, suggests that the 91-kDa polypeptide is also subjected to a second degradation process which is leupeptin-insensitive.

To further localize and to investigate the relationship among the different GPBP-related polypeptides, we isolated matrix and membranes from lysosomes of treated or untreated rats and the corresponding extracts were similarly analyzed. Western blot studies on untreated lysosomal fractions revealed that the major 44-47-kDa polypeptides are located at the lumen and with the exception of the 60-kDa polypeptide, which appeared in some preparations equally distributed between matrix and membrane fractions, the polypeptides other than the 44-47 kDa were found to be membrane-associated components. These findings, in addition to further confirming that the 44-47-kDa GPBP-related polypeptides are integral components of the lysosomes provide some insights on the mechanism of their production. Without being limited to a specific mechanism, we propose that the 91-kDa form exists exclusively attached to the inner face of the lysosomal membrane where it undergoes limited leupeptin-sensitive or leupeptin-insensitive proteolysis to yield the 44-47-kDa or the 32-kDa polypeptides respectively. Leupeptin-sensitive processing is predicted to occur in two principal steps, one with the polypeptide being attached to the membrane yielding a 60-kDa product, and the other requiring release of the 60-kDa intermediate from the membrane and yielding a final products of soluble 44-47-kDa polypeptides. Leupeptin-insensitive processing appears to occur however only on a membrane-bound 91-kDa polypeptide to generate a final product of 32-kDa still bound to the membrane. It remains to be determined whether the 77-kDa polypeptide found in some leupeptin-treated lysosomes represents a proteolytic intermediate or the canonical translation primary product that also enters into this compartment.

All our findings suggest that the 44-47-kDa polypeptides are lysosomal isoforms of GPBP, which mainly derive from non-canonical 91-kDa GPBP. These studies also reveal that, with the exception of the 120-kDa polypeptide, lysosomes contain the enzymatic resources to generate all the GPBP isoforms found in tissue and cell homogenates. This was specifically confirmed by coupling recombinant expression of the mRNA in a cell-free system coupled to

limited proteolysis using rat liver lysosomal extracts (**Fig. 7**). Recombinant expression of mRNA coupled to limited proteolysis produced polypeptides of 77-, 60-, 47-, 44- and 32-kDa revealed that lysosomal proteolysis of primary translation products accounts for all the related polypeptides of lower molecular mass. Similar results were obtained when the proteolytic assays were performed using individual recombinant product (91- or 77-kDa).

Identification of phosphate transfer activity in isolated intact lysosomes and phosphorylation of the 44-kDa GPBP form. The above data suggest that the 44-47 kDa polypeptides are the isoforms of GPBP in the lysosome. We assessed the ability of these polypeptides to transfer phosphates by incubating intact or broken lysosomes with [γ^{32}P]-ATP and further analyzing the mixtures by SDS-PAGE and autoradiography (**Fig.** 8**A**). Untreated, intact lysosomes incubated with isotonic buffer at pH 7 efficiently incorporated ^{32}P at components of 44-47-, 34- and 3.2 to 15-kDa, whereas lysosomal disruption greatly impaired labeling of these materials. To determine the relationship between molecular species that incorporated ^{32}P and GPBP, a protein kinase with a prominent capacity to undergo autophosphorylation, we combined Western blot and autoradiography of SDS-PAGE analysis of phosphorylation mixtures representing control or leupeptin-treated lysosomes (**Fig. 8B**). These studies revealed the presence of labeled components co-migrating with 44-47-kDa polypeptides in control lysosomes in addition to a labeled component of lower molecular mass (~34-kDa) not associated to any immunoreactive species. In general, we observed a fast initial labeling and a gradual reduction in ^{32}P-labeling with the time of incubation. The studies on leupeptin-treated lysosomes revealed a more efficient initial ^{32}P labeling of virtually all the components previously identified in control lysosomes. However, in treated lysosomes the gradual reduction in ^{32}P-labeling of the 44-47-kDa components was accompanied by a gradual labeling of a component that co-migrated with 60-kDa GPBP isoform. Similarly, labeling reduction of non-immunoreactive 34-kDa material was accompanied by increased labeling of a component with higher molecular mass (~38-kDa) that was also not detectable by monoclonal antibodies. These findings suggest that lysosomal GPBP isoforms are either the target of an unknown protein kinase therein or, more likely, the labeling of the GPBP isoforms is the result of an autophosphorylation event. Accordingly, a C-terminal deletion mutant of GPBP with approximately 44-kDa molecular mass (residues 1-299 of GPBP) displayed greater auto-

phosphorylation activity at pH 5 than full sized GPBP, suggesting that lysosomal 44-47-kDa are GPBP isoforms more efficient than 91- and 77-kDa primary products to operate inside the lysosome. In any event, the demonstration of ^{32}P incorporation at the 60- and t44-47-kDa polypeptides provides, to our knowledge, the first evidence for an intrinsic protein kinase activity in lysosomes and points to 44-47-kDa GPBP as the first protein kinase operating inside this cell compartment.

The conformational isomerization of the α3(IV)NC1 domain mainly occurs at the endosomal/lysosomal compartment and depends on GPBP. Recombinant expression of the α3(IV)NC1 domain in human 293 cells results in the synthesis and secretion of multiple polypeptides ranging in size between 22-27-kDa (WO 00/50607 and WO 02/061430). Reduction of disulfide bonds results in a major single molecular species of 29-kDa and multiple derived proteolytic products of lower molecular mass, suggesting that the multiple polypeptides are conformational isoforms (conformers) maintained and stabilized by disulfide bonds that undergo limited proteolysis (**Fig. 9A**). To explore the cell compartment at which the conformational diversification of the α3(IV)NC1 domain occurs, the cells were cultured in the presence of NH_4Cl or leupeptin, lysosomotropic agents that increase the pH and inhibit cysteine proteases respectively. The presence of NH_4Cl reduced conformer production whereas leupeptin inhibited the presence of proteolytic products, suggesting that conformational diversification of the α3(IV)NC1 domain mainly occurs at the endosomal/lysosomal compartment. To explore the role of GPBP in α3(IV)NC1 conformer production, compounds with the capacity to modulate GPBP kinase activity *in vitro* (see below) were used to regulate the corresponding cellular conformer production (**Fig. 9B**). When a positive GPBP modulator (DAB-Am4) was added to the culture medium an efficient increase in conformer production occurred. In contrast, when the compound added was a negative GPBP modulator (Q_2 or Q_4), a reduction in conformer production occurred. All these findings suggest that lysosomal GPBP isoforms are actively involved in the conformational isomerization of the α3(IV)NC1 domain and consequently, the assembly of aberrant α3(IV)NC1 conformers mediating Goodpasture autoantibody production is expected to be a lysosomal event. DAB-Am-4 is a branched polyamine and these compounds have been shown to accumulate in secondary lysosomes (Supattapone S et al. J. Virol (2001) 75, 3453-3461). Fluorescein-labeled Q_2 showed a granular cell distribution with broad co-localization with

GPBP as determined by indirect immunofluorescence approaches on cultured cells. D-amino acid version of Q_2, known to be more refractory to degradation, was significantly more effective inhibiting GPBP cell conformer production.

GPBP, autoimmunity and tissue degeneration. Several lines of evidence support the idea that GPBP is involved in the pathogenesis of other autoimmune diseases: 1) GPBP is preferentially expressed in cells and tissues that are targets of common autoimmune responses; 2) GPBP binds to and phosphorylates other human autoantigens; and 3) Biochemical and immunohistochemical studies show increased levels of GPBP expression in tissues undergoing an autoimmune attack, including cutaneous lupus erythematosus (WO 00/50607) and more recently in cutaneous lesions of patients undergoing systemic lupus erythematosus (SLE).

The autoimmunity response in SLE is due to the involvement of both genetic and environmental factors. New Zealand White (NZW) mice, which do not develop autoimmunity, carry a genetic background that promotes SLE when bred with other mice strains, such as New Zealand Black (NZB). The genetic predisposition of NZW to undergo SLE, and more specifically renal lupus (autoimmune glomerulonephritis), has not been associated with any specific gene(s). In an attempt to relate GPBP with this genetic background, we have performed histological and immunohistochemical studies to address the expression of GPBP in the renal glomerulus of NZW. Our studies suggest that these mice do not undergo a frank autoimmune response. However, 7-9 months after birth they develop a degenerative glomerulopathy that cause glomerulosclerosis and end-stage renal disease (ESRD) with a premature death at 13-14 month of age. Morphologically, this nephropathy presents an evolution with several histological stages. Stage 1, characterized by minimal changes consisting of slight cell proliferation (endothelial and/or mesangial) and light thickening of mesangium. Stage 2, the cell proliferation is moderate and there are collagenous-like deposits at the mesangium which stain with aniline blue, and other deposits of hyaline nature in the subendothelial space that stain with acid fuchsin. Stage 3, characterized by an intense cell proliferation predominantly mesangial with extensive protein deposits that invade subendothelial space (Stage 3a), or endothelial with extensive nodular deposits that invade the mesangium (Stage 3b). At this stage the more characteristic histochemical image is the presence of fuchsinophilic hyaline deposits surrounded by protein deposits of collagenous-like nature. Stage 4, in this stage the glomeruli undergo sclerosis likely

as a consequence of scar organization of the collagenous deposits. When sclerosis results from deposits primarily mesangial this is more diffuse and homogeneous (Stage 4a) than when scar results from reactive fibrosis against subendothelial deposits, in which case this is more nodular and laminated (Stage 4b).

Immunochemical studies performed to address the presence of GPBP revealed that, in contrast to what has been previously described for control mice kidneys (BALB/c and C57BL/6) (WO 00/50607), the NZW kidneys show from moderate to abundant expression of GPBP in tubules and in the interstitial spaces, without significant expression in the glomeruli. However, as glomerular degeneration starts and develops, we detected GPBP expression at the subendothelial space in intimate association with the fuchsinophilic subendothelial material. As the disease progress through Stages 3b and 4b, the expression of GPBP increases substantially.

Furthermore, in an attempt to relate the degenerative process and the production of autoantibodies, we have performed studies to address the presence of immunoglobulin associated with material deposited in the glomerulus of NZW. These studies revealed the presence of linear deposits of immunoglobulins in peripheral capillary loops in a number of glomeruli that varied among individuals (focal and segmentary distribution). As the degenerative process evolved, the number of glomeruli showing linear deposits of immunoglobulins decreases, suggesting that these deposits are a marker of the glomerular structures which are going to undergo degeneration. Consistently, the subendothelial deposits with nodular pattern characteristic of the Stages 3b and 4b showed a high immunoglobulin content.

Finally, in an attempt to determine the nature of the proteinaceous material deposited, we performed histochemical studies using Congo red and thioflavin T, compounds that become adsorbed to the protein deposits of amyloid nature and induce birefringence yellow-green of the polarized light or emit fluorescence, respectively. These studies revealed that the material, which is deposited in the subendothelial space as well as the collagenous material at the mesangium adsorbed these two compounds. However, whereas thioflavin T was excitable and emitted fluorescence, the adsorbed Congo red was unable to induce birefringence to the polarized light. These results indicate that the material deposited shares some structural features with amyloid matter (para-amyloid or amyloid-like).

Our studies suggest that the glomerular degeneration of NZW is primarily caused by an alteration in the folding of certain proteins, which cause aggregation and deposit formation at the

subendothelial space (fuchsinophilic deposits), and at the mesangium (aniline blue stained material). A reactive fibrosis against these deposits is likely the cause of glomerular sclerosis (end-stage renal disease, ESRD). The protein deposits although different than amyloid matter, share with it some structural features. As previously described, we have not found a frank autoimmune response in NZW. However, the presence of immunoglobulins intimately associated with the subendothelial deposits suggest that, as in Goodpasture disease, aberrant conformers induce autoantibody production.

In light of all these findings, we suggest that an aberrant expression of GPBP is part of the genetic background which predispose NZW mice to undergo tissue degeneration (deposition of proteins) and autoantibody production (autoimmune response). Furthermore, the coordinated increase of protein deposits, GPBP, and immunoglobulins at the subendothelial space suggest that the three processes are related.

GPBP is a molecular target for treating diseases mediated by amyloid-like matter.

In an attempt to establish the causal relationship between GPBP activity and the formation of protein deposits in NZW, we have identified modulators of the activity of GPBP and we administered them to these mice.

Branched polymanines (dendrimers) are chemical structures with a large number of peripheral reactive amines which are commonly used to be substituted by one or more chemical groups to increase their presence at the molecular surface and thus enhance their biological/therapeutic activity. We have found that branched polyamine of first generation [Sigma product numbers 46,069-9: polypropylenimine tetraamine dendrimer (DAB-Am-4)] is a potent activator of GPBP kinase activity in vitro and α3(IV)NC1 conformer production in cultured cells (see above). After performing toxicity assays in mice, we administered non-toxic doses of DAB-Am-4 to 4-6 month-old NZW mice, and we studied its consequences in the progression of the glomerulopathy. These studies revealed that DAB-Am-4 caused an acceleration of the degenerative process, resulting in premature glomerulosclerosis at 7-9 month of age. Whereas in the natural progression of the disease, the morphological pattern more frequently found was that through Stages 3a and 4a, the treatment induces almost constantly a progression through Stages 3b and 4b with abundant presence of GPBP intimately associated with protein deposits. These data suggest that an augmented activity of GPBP is causally related

with the progression of the degenerative process towards sclerosis and ESRD. In trials on control mice (C57BL/6) we have not observed histological changes of relevance due to administration of DAB-Am-4, suggesting that the capacity for DAB-Am-4 to induce glomerular sclerosis depends mainly on the NZW genetic background which possibly involves aberrant activation/expression of GPBP.

To verify that an induction of GPBP in the genetic context of NZW is responsible for the degenerative process, NZW mice were treated with DAB-Am-4 or with DAB-Am-4 and Q_2, a synthetic peptide (LATLSHCIELMVKR) (SEQ ID NO:90) that encompasses a motif of GPBP for self-interaction in two-hybrid studies, and thus suspected to be critical for GPBP aggregation, that efficiently inhibits GPBP kinase activity in vitro and α3(IV)NC1 conformer production in cultured cells (see above and below sections). The treatment with Q_2 sharply reduced the material deposited in the glomerulus of NZW of suspected collagenous nature although it was shown to be unable to reduce the presence of fuchsinophilic material at the subendothelial space. A D-amino acid version of Q_2 was significantly more effective than the L-amino acid version consistent with its more potent inhibitory activity on GPBP kinase activity in vitro and on cellular conformer production.

One way to interpret these findings is that Q_2 efficiently blocks progression from Stage 3b to 4b during disease induction by DAB-Am-4. In other words, the presence of abundant fuchsinophilic material in Q_2 treated mice is suspected to be caused by the lack of fibrotic reaction which substitutes or masks fuchsinophilic material during disease progression. This results in glomeruli virtually devoid of fibrotic reaction that causes ESRD.

When we assessed thioflavin T or Congo red staining, we found that the material which stains with acid fuchsin, contrary to the homologous material at Stage 3b in natural disease or in DAB-Am-4 induced disease, did not adsorb either compound, suggesting that by inhibiting GPBP, Q_2 efficiently inhibited amyloid-like matter formation in NZW. Finally, this specific effect on protein deposit structure could be responsible for an attenuated fibrotic reaction, and the lack of progression towards glomerular sclerosis and ESRD.

Identification of multiple compounds that modulate GPBP kinase activity in vitro and α3(IV)NC1 conformer production in culture cells. To determine the role of GPBP in the conformational diversification of the α3(IV)NC1 domain, we have first identified and

characterized different modulators of kinase activity of GPBP in vitro, and later we have used them to modulate conformer production in cultured cells.

We have reported that GPBP self-interacts and that aggregation regulates kinase activity (WO 00/50607). By combining a yeast two-hybrid system and cDNA deletion mutants of GPBP, we have identified a five-residue (SHCIE) (SEQ ID NO:39) and a ten residue (EKTAGKPILF) (SEQ ID NO:45) motifs in the GPBP amino acid sequence that are critical for self-interaction. A synthetic peptide representing the five-residue motif and flanking regions (LATLSHCIELMVKR, called here Q_2) (SEQ ID NO:90) efficiently inhibited GPBP autophosphorylation, whereas a synthetic peptide representing the ten-residue motif (EKTAGKPILF, called here Q_4) (SEQ ID NO:45) inhibited GPBP autophosphorylation in a more limited manner. Furthermore, when these peptides where separately added to the culture media of cells expressing α3(IV)NC1, Q_2 was more effective inhibiting cell conformer production than Q_4 which had a more limited inhibitory effect (see above). A D-amino acid version of Q_2 (Q_{2D}) was more efficient inhibiting GPBP autophosphorylation and conformer cell production than the corresponding L-amino acid version (Q_{2L}).

We have also assayed a number of protein kinase inhibitors (Calbiochem Cat No 539572) and found that staurosporine (a broad range Ser/Thr kinase inhibitor) and KN93, 2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine [an inhibitor highly specific for Ca^{2+}Calmodulin-dependent protein kinase II (CaM kinase II)], efficiently impaired GPBP kinase activity in vitro. KN62, 1-[N,O-bis-(5-Isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine [another specific inhibitor for CaM kinase II (Sigma Cat No I2142)], inhibited GPBP kinase activity in vitro to a larger extent than KN93, which in contrast is known to be a more potent inhibitor than KN62 for CaM kinase II. These organic compounds are thought to inhibit CaM kinase II by interfering with the binding of the Ca^{2+}Calmodulin activator complex to the kinase. In vitro studies consistently revealed that calmodulin inhibited GPBP kinase activity in a Ca^{2+} dependent manner, suggesting that GPBP contains a binding site for Ca^{2+}Calmodulin similar to that of CaM kinase II, although the consequences of binding on kinase activity were different in each case. Finally, when KN62 and KN93 were separately added to the culture media of α3(IV)NC1-secreting cells, we found that these compounds reduced cell conformer production. These findings, in addition to identify potential compounds for modulating GPBP kinase activity and α3(IV)NC1 cell conformer

production, uncover important features of allosteric regulation of GPBP, and reveal that GPBP has catalytic features that are similar to CaM kinase II. CaM kinase II also requires self-aggregation to be functional, and Q_2 efficiently inhibited CaM kinase II, suggesting that the interacting motifs of these two kinases are structurally related. Consistent with this idea aggregatable CaM kinase II contains a highly homologous five-residues motif, SHCIQ (SEQ ID NO:40), not present in non-aggregatable CaM kinases.

Although Q_4 appeared to be a poor inhibitor under experimental conditions in which Q_2 efficiently inhibited GPBP kinase activity, this peptide showed synergistic inhibitory effect on GPBP kinase activity with KN62 (+++), Ca^{2+}CaM (++) and Q_2 (+). Similarly we found inhibitory synergism on GPBP kinase activity when combining KN62 and Ca^{2+}CaM (+++), whereas no cooperative inhibitory effect was observable when combining Q_2 and Ca^{2+}CaM or Q_2 and KN62.

In contrast to the positive regulatory effect of DAB-Am-4, fourth generation of branched polyamines (DAB-Am-32) (Sigma Chemical Co. product number 46,908-4) efficiently inhibited GPBP protein kinase activity. This compound has been shown to accumulate in the lysosomes and exert a curative effect on prion infected cultured cells (Supattapone S. *et al.* J. Virol(2001) 75, 3453-3461).

These data, in addition to provide experimental evidence for the role of GPBP in the conformational isomerization of the α3(IV)NC1 domain in vitro and ex vivo, report the first repertoire of lead compounds to be use for treatment of autoimmune diseases and protein deposit-mediated disorders.

GPBP is a potential molecular target for treating amyloidosis. GPBP represents the first example of a molecular enzyme showing kinase and polypeptide conformation isomerase activity (WO 00/50607 and WO 02/061430). We have shown that a 91-kDa isoform of GPBP resulting from non-canonical mRNA translation enters the lysosome and undergoes processing to yield a 44-47-kDa product, which in turn represents a GPBP isoforms that are integral components of this cell compartment. The evidence suggests that in this acidic environment, GPBP can transfer phosphate and therefore conceivably could also catalyze conformational isomerization reactions.

An important number of human degenerative diseases, including Alzheimer's and prion

diseases, are mediated by the presence of aggregates (amyloid matter) made of non-soluble conformational versions of specific cellular components ($A\beta_{1-42}$ and PrP^{Sc}, respectively). The conformational isomerization of prion protein, PrP, which represents the best known process involving conformational shift of a polypeptide (PrP^{C} to PrP^{Sc}), occurs after anchorage of the PrP^{C} to the plasma membrane during internalization through the endosomal pathway, and is suspected to be assisted by a chaperone-like protein (Protein X). N terminal trimming of PrP^{Sc} to produce PrP 27-30, the material found in amyloid deposits of infected cultured cells, occurs in secondary lysosomes, suggesting that this cell compartment is critically involved in the pathogenesis prion diseases (Prusiner, S.B. (2001) N England J Med 344, 1516-1526).

Our data suggest that the α3(IV)NC1 conformers are secreted and undergo proteolysis via endosomal/lysosomal pathways, and that specific modulators of GPBP regulate α3(IV)NC1 conformer production in cultured cells. All of the above suggests that α3(IV)NC1 and PrP share secretory and degradation pathways, and, therefore GPBP may catalyze similar conformational isomerization reactions on PrP.

Fourth generation branched polyamines DAB-Am-32 have been reported to accumulate in lysosomes and to cure prion infected cells. DAB-Am-32 strongly inhibited GPBP in vitro at concentrations at which DAB-Am-4 caused induction. This suggests that, as for para-amyloid matter formation, amyloid matter deposition requires GPBP action and also suggests that one of the mechanisms by which DAB-Am-32 eliminates amyloid matter in prion infected cells involves inhibition of GPBP at the lysosomal compartment. This results point to GPBP as a potential candidate for Protein X activity.

The results above suggest that the reported curative effect of branched polyamines on prion infected cells may be due in part to inhibition of otherwise active lysosomal GPBP, thus implicating GPBP as a therapeutic target in prion-mediated disorders.

GPBP binds to PrP^{C} in vitro. To show the biological relationship between GPBP and proteins that promote amyloid matter formation when they undergo conformational degeneration, the interaction between GPBP and PrP^{C} was assessed in far Western assays using cellular extracts of primary cultures of rat cerebelar neurons and recombinant human GPBP. GPBP bound to a limited number of polypeptides of different sizes all of which were recognized by specific antibodies against PrP^{C} (Santa Cruz Biotech Ca# SC7693) (**Fig. 10**). The presence of

GPBP in these cells was further demonstrated by Western blot analysis of the corresponding cell extracts using specific antibodies. Furthermore, we used recombinant material representing human GPBP and bovine PrP^C in specific far Western and phosphorylation studies and found that GPBP interacts with PrP^C and when incubated in the presence of $[\gamma^{32}P]$-ATP transferred phosphates to PrP as a result of this interaction (**Fig. 10**).

Human GPBP aggregates with bovine PrP^C. To explore further the pathway of complex formation between PrP^C and GPBP, we used spectroscopy methods. Light scattering at 90° was measured for the aggregation kinetic assays. Upon addition of GPBP at a PrP^C solution, aggregation occurred and could be monitored by light scattering. The complex formation is independent of the time course of protein addition since the same increase in the light scattering signal is obtained when a PrP^C solution is added to a GPBP solution initially placed in the measurement cell. To ascertain whether the different versions of inhibitory Q_2 peptide (L-amino acid and D-amino acid versions of Q_2 peptide -Q_{2L}, Q_{2D} respectively- and Q_{2Lr}, an inactive scrambled peptide with the same amino acid composition than Q_{2L}) could affect GPBP-PrP^C complex formation in a similar manner than they affected kinase activity, we monitored aggregation in the presence of each individual peptide. Upon addition of GPBP to a PrP^C solution containing 100 μM of Q_{2L} , GPBP-PrP^C complex formation was efficiently inhibited. The inactive peptide Q_{2Lr} had no effect on complex formation at these concentrations, whereas the more potent Q_{2D} peptide at 20 μM fully inhibited GPBP-PrP^C complex formation.

Aggregation of GPBP and PrP^C depends on structural requirements for Protein X interaction. Interaction of PrP^C and Protein X is expected to occur through a defined number of residues at the C terminal region of PrP which comprises the Protein X binding site (Kaneko, K, et al., (1997) Proc. Natl. Acad. Sci. *USA* 94, 10069-10074). We have performed recombinant expression and immunoprecipitation studies in an attempt to first assess whether GPBP-PrP complex formation is mediated by a Protein X-type interaction. We have used specific antibodies recognizing FLAG-tag sequence only present in recombinant GPBP to precipitate co-expressed recombinant human PrP. FLAG-specific antibodies efficiently precipitated FLAG-GPBP along with PrP, suggesting that FLAG antibodies-GPBP-PrP form a precipitable ternary complex and that GPBP and PrP interact in the cellular environment. When immunoprecipitations were done

on cell extracts expressing GPBP and individual PrP mutants we observed that mutants expected to alter Protein X binding site were precipitated by FLAG-antibodies in a much less efficiency than mutants not involving these residues, which showed a similar capacity to undergo precipitation than PrP representing wild type sequence. In **Fig. 11** we illustrate a comparative study between PrP and PrP^{E168R}, a human PrP mutant in which a residue proposed to be part of Protein X epitope, E^{168}, has been replaced by R to generate a PrP^{C} mutant non-susceptible for PrP^{Sc} conversion (Kaneko, K, et al., (1997) Proc. Natl. Acad. Sci. *USA* 94, 10069-10074). Similar results were obtained with functionally homologous PrP^{Q172R} and PrP^{E219K} mutants but not with two independent non-functionally related PrP^{R220A} and PrP^{R228A} mutants .

GPBP promotes conformational changes in PrP. A widely used method to monitor conformational alterations in PrP^{C} relevant to pathogenesis comprises determination of the number of related polypeptides being expressed by the cell and their soluble or precipitable condition. In general, PrP^{C} is expressed inside the cell as a highly soluble single polypeptide and an increased number of polypeptides with poor solubility is characteristic of PrP^{Sc} and other non-physiological conformational forms of PrP such as PrP^{Res} or $PrP^{Sc\text{-}like}$, and more recent data suggest that inside the cell insoluble conformers of PrP are continuously being produced and cleared and that the levels of these conformers at the steady state reflects the dynamics of these two opposite processes (Ma, J. and Lindquist (2002) Science 298, 1785- 1788). We have used inhibitors and activators of GPBP to regulate the levels of precipitable recombinant human PrP polypeptides in cultured cells **(Fig. 12)**. The presence of DAB-Am-4 in the culture media of cell expressing recombinant PrP efficiently induced the expression of non-soluble precipitable PrP polypeptides, whereas the presence of DAB-Am-32 efficiently inhibited expression of non-soluble precipitable PrP polypeptides. Similarly, we have generated and used a number of mRNA silencers to regulate the level of expression of non-soluble PrP polypeptides and found that individual mRNA silencers down-regulated non-soluble PrP expression to an extent consistent with the capacity displayed by each individual mRNA silencer to impair endogenous non-canonical expression of GPBP. In **Fig. 13** we illustrate a comparative study using a non-relevant silencer (C) and two specific silencers (1,2) with higher (1) or lower (2) capacity to reduce 91- and 120-kDa GPBP expression (see **Fig. 4**, lanes 5 and 7, respectively).

GPBP bind to $A\beta_{1\text{-}42}$

Several lines of evidence suggest that senile plaques in Alzheimer's disease derive from neurons that have undergone degeneration primarily caused by amyloid deposition of $A\beta_{1-42}$ at secondary lysosomes (Nixon, et al., (2000) Neurochem Res 25, 1161-1172; Andrea, MR, et al (2001) 38, 120-134). Conceivably, a similar mechanism to that proposed above to be mediating amyloid matter formation in prion diseases could be mediating amyloid matter deposition in Alzheimer's disease. To assess this possibility the capacity of GPBP to bind to $A\beta_{1-42}$ was assessed in specific far Western studies (**Fig. 14**). Recombinant human GPBP displayed high affinity for a synthetic polypeptide representing $A\beta_{1-42}$, whereas in the same assay conditions GPBP did not display binding capacity towards a synthetic peptide representing the non-phosphorylable N terminal region of bovine $\alpha3$(IV)NC1 . Incubation of GPBP with $A\beta_{1-42}$ in the presence of $[\gamma^{32}P]$ATP did not result in ^{32}P-labeling of synthetic polypeptide, suggesting that although $A\beta_{1-42}$ contains sites for GPBP molecular recognition, it does not harbor GPBP phosphorylation sites. Consequently, $A\beta_{1-42}$ perhaps represents a substrate of GPBP for a conformational catalysis in which phosphate transfer of protein substrate is not required. The latter suggests that GPBP-mediated conformational catalysis on protein substrates can occur in a phosphorylation-dependent or independent manner, or that conformational catalysis can be performed on phosphorylated or non-phosphorylated substrates. Consistently, GPBP bound with more affinity to recombinant proteins representing phosphorylated version of human autoantigens (Goodpasture antigen and myelin basic protein) at specific Ser that conform phosphorylation sites for GPBP (Ser^9 and Ser^8, respectively), suggesting that the phosphorylated products are not the end product of GPBP catalysis, but they are the substrate for a conformational isomerization and supramolecular assembly catalysis.

In full our data provide the first experimental support for GPBP being the chaperone-like molecular enzyme suspected to be involved in PrP^C to PrP^{Sc} conformational isomerization in the pathogenesis of prion diseases, and also represents the first molecular link between two previously unrelated processes, tissue degeneration mediated by amyloid and para-amyloid matter deposition and autoimmunity.

DISCUSSION

Autoimmune diseases comprise a large number of disorders mediated by an immune attack against self-components (autoantigens) as a result of a failure in the mechanisms of

immune tolerance. When autoantigens are administered to animal models they have the peculiar capacity to engage the immune system in a response that mimics the natural disease revealing that these components display biological features of immunological relevance. Consequently, certain alterations in autoantigen biology could have an important impact in their immunological recognition, thus triggering an immune response. For these reasons, understanding autoantigen biology is necessary to design an appropriate molecular model for autoimmune disorders.

By studying Goodpasture (GP) disease we have provided new insights into the molecular mechanism of autoimmune disorders. GP disease is characterized by the coexistence of glomerulonephritis and lung hemorrhage caused by an immune attack that is mediated by circulating autoantibodies, which deposit in a linear manner in the glomerular and alveolar basement membranes. The autoantibodies are directed against the C terminal non-collagenous domain (NC1) of the α3 chain of collagen IV, α3(IV)NC1 domain, also called the Goodpasture antigen. Collagen IV is composed of six α-chains that exhibit a high degree of homology which is more evident at the NC1 domain. However, only the α3(IV)NC1 domain has been shown to induce Goodpasture syndrome in animals models, and only the human α3(IV)NC1 domain has been implicated in a common natural autoimmune response. Comparative structural studies identified a highly divergent region at the N terminus of the α3(IV)NC1 domain which undergoes phosphorylation by cAMP-dependent protein kinase and also by GPBP. α3(IV)NC1 domain is purified from natural sources as a set of conformational isomers (conformers) with differential phosphoserine content. The more abundant α3(IV)NC1 conformer, which likely represents the native conformation, is virtually devoid of phosphoserine, whereas the less abundant α3(IV)NC1 conformers, likely representing derived alternative conformations, display the highest degree of phosphoserine content. These data suggested that phosphorylation is part of the strategy used by cells to generated alternative protein conformations (WO 00/50607; WO 02/061430).

Other biological consequences associated with phosphorylation of the α3(IV)NC1 domain include regulation of α3(IV)NC1 domain aggregation. In the absence of ATP, GPBP displays the capacity to catalyze a physiological aggregation of the α3(IV)NC1 domain (disulfide-dependent oligomerization) by a process involving conformational isomerization (WO 02/061430). This data indicate that GPBP possesses a conformational isomerase activity

independent of its kinase activity that is critical for a broader enzymatic catalysis, resulting in assembly of a protein substrate into a quaternary structure.

The relationship between GPBP and autoimmune pathogenesis was initially established by showing (a) elevated levels of GPBP in Goodpasture patients; (b) *In vitro*, GPBP catalyzes the production of α3(IV)NC1 conformers that are found in patient kidneys but not control kidneys; and (c) The presence of aberrant α3(IV)NC1 conformers in patient kidneys that are specifically recognized by pathogenic autoantibodies. (WO 02/061430) These results led to a new model of autoimmune disease, wherein the autoimmune response is considered a legitimate reaction of the immune system against aberrant conformations of an autoantigen (such as the α3(IV)NC1 in GP disease), which assemble and for which the immune system has not established a tolerance (WO 00/50607; WO 02/061430).

These observations identify critical and exclusive biological features in a human autoantigen (the α3(IV)NC1) that do not have a counterpart in homologous domains (the other type IV collagen NC1 domains) which are not autoantigens, and represent a new strategy to study the molecular basis of a human exclusive disease. Recent studies performed in our laboratory have identified the presence of a serine residue in the N terminal region of myelin basic protein ("MBP") that is structurally and functionally similar to that found in the α3(IV)NC1 (WO 02/061430). Myelin basic protein is a major autoantigen in an autoimmune response mediating multiple sclerosis, which, like Goodpasture disease, is an exclusively human autoimmune disease. Recent studies further demonstrated that phosphorylation of MBP plays a critical role in regulating its conformation and have identified conformational-dependent differences in the proteolytical susceptibility of myelin basic protein from control and patients affected by multiple sclerosis (data not shown). These data represent a strong validation of our model of autoimmune disease, based on the biology of two unrelated human autoantigens and autoimmune disorders, and suggest that a common autoimmune pathogenic mechanism is emerging in which GPBP plays a central role. In this mechanism, human autoantigens are polypeptides with the capacity to bind to GPBP and, as a consequence of such a binding, undergo phosphorylation and conformational isomerization, which makes these polypeptides vulnerable to an aberrant catalysis and production of non-tolerized conformers (WO 00/50607; WO 02/061430).

The high phosphorylability of myelin basic protein *in vitro* contrasts with its low content in Ser(P) residues, suggesting that (a) endogenous phosphorylation of this autoantigen is highly regulated; and (b) there are multiple MBP species with different degree of phosphorylation [Eichberg, J., & S. Iyer , Neurochem Res 21, 527-535 (1996)]. Conceivably, the sequential phosphorylation and dephosphorylation of specific sites on autoantigens, such as those identified at the N terminus in myelin basic protein and in the human α3(IV)NC1 domain, could generate a heterogenous population of molecules or conformers for supramolecular assembly, and an alteration in the homeostasis of phosphorylation events could result in the assembly of aberrant non-tolerized conformers in the corresponding quaternary structure of the autoantigens.

GPBP displays a number of biological features to be considered a good candidate as a pivotal component of the cellular machinery catalyzing the supramolecular assembly of autoantigens and inducing immune response during autoimmune pathogenesis. For example: (1) GPBP phosphorylates homologous sites in two different human autoantigens and targets other human autoantigens; (2) The GPBP phosphorylation sites in myelin basic protein and Goodpasture antigen play a conformational regulatory role; (3) GPBP binds preferentially to recombinant species representing the phosphorylated versions at these sites, suggesting that the phosphorylated versions are not only the product of a phosphate transfer reaction, but are also the substrate of an additional catalysis that includes conformational isomerization and supramolecular assembly; (4) Immunochemical studies show that GPBP is present in tissue, cellular and subcellular localizations that are common targets of autoimmune responses; (5) Increased levels of GPBP relative to its alternatively spliced isoform, GPBPΔ26, are found in several autoimmune conditions (WO 00/50607; WO 02/061430 and data not shown).

To further establish the role of GPBP in autoimmune pathogenesis, a major issue is to determine the mechanism by which GPBP is delivered to such a broad number of subcellular localizations. Proteins can be synthesized at free ribosomes (proteins to be resident at the cytosol or to be further transported to, for example, nucleus, mitochondria or peroxisome) or at ribosomes associated with ER (proteins that enter into the secretory pathway and end up being either ER, Golgi apparatus, lysosomes and plasma membrane resident, or secreted to the extracellular matrix). There are proteases present in all these locations, and there are many examples in which primary translation products undergo proteolysis to render shorter biologically active polypeptides.

In the cell, protein sorting is accomplished via a number of signal sequences, many of which have been characterized. However, there are increasing examples of non-canonical mechanisms for cellular protein sorting.

By studying the cellular expression of GPBP, we have established that the cell expresses at least seven GPBP-related polypeptides of 120-, 91-, 77-, 60-, 44-47-, 32-kDa. With the exception of 120-kDa GPBP, whose origin is not certain, the rest can be generated by limited proteolysis of the 91-kDa polypeptide, as shown herein. We present evidence suggesting that the 91 kDa GPBP is a non-canonical translation product of GPBP mRNA. The evidence presented herein also suggests that 91 kDa GPBP enters into the secretory pathway and undergoes processing to produce GPBP isoforms of lower molecular mass that can be found in the ER, Golgi apparatus, lysosomes and plasma membrane.

Confocal studies performed in our laboratory show a major co-localization of GPBP and Goodpasture antigen in human glomerulus suggesting the presence of GPBP in basement membranes. In contrast, our evidence from recombinant expression studies suggests that canonical 77-kDa polypeptide is essentially cytosolic (data not shown). However, subfractioning studies show that at the cellular steady state the levels of canonical primary product are negligible and only a major derived product of 60-kDa can be detected, suggesting that the 77-kDa primary product, if it is expressed, undergoes an efficient processing to a lower molecular mass isoform. The mechanisms for GPBP transport to the nucleus and mitochondria (WO 00/50607; WO 02/061430) remain to be verified, although our data suggests that certain non-canonical translation products may provide the requisite targeting signals for such localization.

Recombinant expression shows that the 5'-UTR contains multiple non-canonical sites for translation initiation that display a 5' to 3' hierarchy. Based on sequence analysis and using programs that predict subcellular localization, the ORF in Δ102 contains a canonical signal peptide sequence to entry into the secretory pathway (residues 1-46). This signal peptide is immediately followed by a signal for nuclear localization (residues 47-50) and another for mitochondrial destination (residues 52-56), in turn, suggesting that by varying transcription initiation site the cell may regulate the expression of non-canonical polypeptides that are destined for the secretory pathway (ER/Golgi apparatus/lysosomes/plasma membrane/extracellular matrix), nuclear o mitochondrial whereas only canonical translation would generate a genuine cytosolic polypeptide. Furthermore, GPBP also displays two other

potential mechanism to reach nuclear environment: (a) GPBP contains a bipartite nuclear localization signal; and (b) GPBP binds to a family of transcription factors that could shuttle the protein into the nucleus (WO 03/048193).

The cellular expression of GPBPΔ26 was also explored using Mab14, a monoclonal antibody recognizing both GPBP and GPBPΔ26 recombinant counterparts. Mab14 reacted with a single 77-kDa cytosolic polypeptide and did not show significant reactivity towards polypeptides reacting with Mab6. The specificity of these Mab14 antibodies was confirmed by demonstrating that GPBP/GPBPΔ26 silencers reduced the expression of 77-kDa polypeptide to similar extent than 91- and 120-kDa polypeptides that only reacted with Mab6. These results suggest that the 77-kDa polypeptide is primarily GPBPΔ26 and cytosolic, whereas non-canonical polypeptides are mainly GPBP, and virtually ubiquitous.

In summary, our data suggest that for native cellular expression Mab14 is an immunological probe for GPBPΔ26 whereas Mab6 is an immunological probe for GPBP-related polypeptides.

Our findings suggest that GPBP is an integral component of the endosomal-lysosomal pathway which activity is regulated in part by a catepsin-dependent processing, a biological strategy described for other enzymes (Pham, C. T., & T. J. Ley, (1999). Proc Natl Acad Sci U S A 96(15): 8627-8632). These proteases are critical in processing proteins entering the endosomal pathway, and for producing peptides that are presented through MHC class II (Chapman, H. A., (1998) Curr Opin Immunol 10(1): 93-102). Disturbances of lysosomal environment in a general manner, such as modifying the pH using compounds as chloroquine, or in a specific manner using catepsin inhibitors such as leupeptin, have been shown to alter peptide presentation by MHC class II (Demotz, S., P. M. Matricardi, C. Irle, P. Panina, A. Lanzavecchia, & G. Corradin, (1989) J Immunol 143(12): 3881-3886; Turk, V., B. Turk, & D. Turk, (2001) EMBO J 20(17): 4629-4633). We have shown herein that leupeptin treatment substantially alters lysosomal processing of GPBP and therefore also likely induces an alteration in GPBP activity, which in turn suggests that altered peptide presentation and altered GPBP activity may be related and perhaps critical in autoimmune pathogenesis, which necessarily requires aberrant peptide presentation to be effective.

A feature common to many degenerative diseases is the formation of deposits of specific polypeptides. Where and how these deposits appear is highly specific and tightly related with

pathogenesis. The deposits can be nuclear inclusion bodies, as in cerebelar ataxia, or be at the ER lumen, such as in some degenerative disease affecting liver and neurons, or be cytoplasmic inclusion bodies, as in Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis; and endosomal-lysosomal, as in Alzheimer's disease, prion diseases, and type II diabetes. GPBP is an ubiquitous protein that has been independently related to conformational catalysis of substrate proteins (WO 00/50607; WO 02/061430) and in the formation of protein deposits in animal models that develop a degenerative nephropaty associated to an autoimmune response. Consequently the finding that GPBP interacts with PrP and $A\beta_{1-42}$ two polypeptides that undergo conformational alteration and form amyloid deposits in prion and Alzheimer's disease, respectively, represents strong evidence for GPBP being involved in the pathogenesis of these degenerative disorders. More specifically a protein resident in the endosomal-lysosomal pathway named Protein X has been proposed to bind to PrP and catalyze the conformational transition from PrP^{C} to Prp^{Sc} (Prusiner, S. B., (1998). "Prions." Proc Natl Acad Sci U S A 95(23): 13363-13383.). Here we present evidence indicating that GPBP binds to PrP in a Protein X fashion, phosphorylates PrP, forms aggregates with it and, as a consequence of this interaction, PrP undergoes conformational changes that renders PrP highly insoluble and precipitable. To our knowledge, GPBP represents the best molecular candidate to be Protein X in prion diseases as well as to perform a similar catalytical role in other protein deposit-mediated human disease.

A major obstacle when studying the molecular basis of degenerative or autoimmune diseases is the almost general consensus that any protein can be an autoantigen or to conformationally degenerate and form deposits. According to this view, the establishment of an autoimmune response represents a non-legitimate immune reaction, while conformational degeneration is thought to represent a stage that any polypeptide chain can achieve if the environment is appropriately altered. However, this view cannot explain the principal fact that only a very limited number of cellular components can be autoantigenic or can form deposits that cause tissue degeneration, indicating that autoantigens and deposit-forming polypeptides share biological features. Our studies suggest that a common biological feature of autoantigens is being a substrate of an enzymatic strategy to form quaternary structures in which GPBP plays a central role and the protein substrate undergoes conformational isomerization. Our results regarding polypeptides that, like PrP and $A\beta_{1-42}$, conformationally degenerate and form deposits, suggest that they are also substrates of GPBP and its catalytic action is required for deposit formation.

While the present invention is not limited to a specific mechanism, we propose that GPBP is a novel molecular enzyme that binds to and phosphorylates protein substrates as part of an enzymatic strategy in which conformational catalysis of protein substrates occur during their supramolecular assembly (quaternary structure). Alterations in its performance produce aberrant conformers that are soluble and induce autoimmunity, or are insoluble and form deposits of amyloid or para-amyloid nature that cause tissue degeneration.